The mechanism of opiorphin-induced experimental priapism in rats involves activation of the polyamine synthetic pathway

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Kanika ND, Tar M, Tong Y, Kuppam DS, Melman A, Davies KP. The mechanism of opiorphin-induced experimental priapism in rats involves activation of the polyamine synthetic pathway. Am J Physiol Cell Physiol 297: C916–C927, 2009. First published August 5, 2009; doi:10.1152/ajpcell.00656.2008.—Intracorporal injection of plasmids encoding opiorphins into retired breeder rats can result in animals developing a priapic-like condition. Microarray analysis demonstrated that following intracorporal gene transfer of plasmids expressing opiorphins the most significantly upregulated gene in corporal tissue was the ornithine decarboxylase gene (ODC). Quantitative RT-PCR confirmed the upregulation of ODC, as well as other genes involved in polyamine synthesis, such as arginase-I and -II, polyamine oxidase, spermidine synthase, spermidine acetyltransferase (SAT), and S-adenosylmethionine decarboxylase. Western blot analysis demonstrated upregulation of arginase-I and -II, ODC, and SAT at the protein level. Levels of the polyamine putrescine were upregulated in animals treated with opiorphin-expressing plasmids compared with controls. A direct role for the upregulation of polyamine synthesis in the development of the priapic-like condition was supported by the observation that the ODC inhibitor 1,3-diaminopropane, when added to the drinking water of animals treated with plasmids expressing opiorphins, prevented experimental priapism. We also demonstrate that in sickle cell mice, another model of priapism, there is increased expression of the mouse opiorphin homologue in corporal tissue compared with the background strain at a life stage prior to evidence of priapism. At a life stage when there is onset of priapism, there is increased expression of the enzymes involved in polyamine synthesis (ODC and arginase-I and -II). Our results suggest that the upregulation of enzymes involved in the polyamine synthetic pathway may play a role in the development of experimental priapism and represent a target for the prevention of priapism.

PRIAPISM IS A PERSISTENT, often painful, penile erection that continues hours beyond, or is unrelated to, sexual stimulation (23). It can lead to progressive fibrosis of the erectile tissue and ultimately result in erectile dysfunction (10). It is quite prevalent in certain populations; for example, its incidence in male patients with sickle cell disease is ~40% (11). Several groups are researching the molecular mechanisms that result in priapism in the hope that a better understanding of the disease would identify targets useful for the development of pharmacotherapies (41). Pioneering work to elucidate the molecular mechanisms that lead to priapism developed from the unexpected observation that mice genetically engineered with deletion of the endothelial nitric oxide synthase gene (eNOS; eNOS−/− knockout mice) exhibited excessive erectile tendencies (5). Later work demonstrated that in the corpora of the eNOS−/− mice there was reduced phosphodiesterase 5 (PDE5) expression (6). It was suggested that following sexual stimulation, resulting in increased release of neuronal NO, large amounts of cGMP are produced and because of insufficient functional phosphodiesterase (PDE5) available to degrade the cyclic nucleotide this results in excessive erectile tissue relaxation. A significant role of both cGMP and cAMP in the development of priapism was recently demonstrated by Mi et al. (22), who investigated the molecular mechanisms by which mice deficient in adenosine deaminase (Ada−/− mice) develop a priapic-like condition. They demonstrated that the priapic-like condition in Ada−/− mice required AdoHcy adenosine receptor-mediated cAMP and cGMP induction.

We have previously reported that plasmids expressing the genes encoding opiorphins (pVAX-Vcsa1, expressing the rat opiorphin homologue, called sialorphin, and plasmids expressing the human homologues, pVAX-hSMR3A/B or pVAX-ProL1) can cause a priapic-like condition when intracorporally injected into retired breeder rats (35–37). We have demonstrated that the rat opiorphin homologue (sialorphin) can increase the rate of corporal smooth muscle relaxation by C-type natriuretic peptide (9). We proposed that this mechanism is related to the activity of the opiorphins as potent endogenous neutral endopeptidase (NEP) inhibitors, protecting bound peptide agonists from proteolysis by NEP (31). We recently reported that changes in expression of Vcsa1 can result in modulation of G protein-coupled receptor (GPCR) expression in corporal smooth muscle cells (38). This could in turn affect cellular processes, or expression of other genes, through changes in GPCR activity and thereby cAMP or cGMP levels.

To gain insight into the molecular mechanism by which opiorphin expression might induce a priapic-like condition in the rat, we determined global changes in gene expression using microarray analysis following intracorporal gene transfer of plasmids expressing opiorphins (pVAX-Vcsa1 and pVAX-hSMR3B). We determined that one of the most upregulated genes encoded ornithine decarboxylase (ODC). Treating animals with an ODC inhibitor prevented priapism caused by intracorporal injection of plasmids expressing opiorphins, suggesting that the upregulation of ODC directly contributes to the development of opiorphin-induced experimental priapism. We also determined whether there were similar changes in key enzymes of the polyamine synthetic pathways of a well-accepted animal model of priapism, the sickle cell mouse (16).

MATERIALS AND METHODS

Animal models. Sprague-Dawley “retired breeder” rats (9–10 mo, weighing >500 g) were obtained from Charles River (Wilmington, MA). Intracorporal injection of 100 µg plasmids for determining gene
and protein expression (pVAX-Vcsa1, pVAX-hSMR3B, pVAX, or pVAX-hSlo gene) was previously described (35–37). Briefly, the rats were anesthetized with pentobarbital sodium (35 mg/kg ip). An incision was made through the perineum, and a “window” was made in the ischiocavernous muscle to identify the crura. All microinjections, using an insulin syringe, consisted of a bolus injection of naked plasmid DNA into the lumen of the crura. The final volume of all microinjections was 150 μl. For the ODC inhibition studies, animals either received water containing 1,3-diaminopropane (2.5 g/l; Sigma, St. Louis, MO) throughout the experiment (beginning 1 wk prior to plasmid injection), and controls were given water alone. This treatment has been previously demonstrated to reduce ODC activity in the bladder of rats by >60% (33). Transgenic, 5-wk-old, sickle cell mice were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained in the Albert Einstein Animal Facility till the life stage to be investigated (5, 10–12, and >12 wk old) was attained. Age-matched C57BL/6 animals were used as controls. Numbers of animals for each experiment are described in figure and table legends. All animal studies were approved by the Institute of Animal Studies at Albert Einstein College of Medicine.

Measurement of ICP/BP. One week following intracorporal injection of 100 μg plasmids (pVAX-Vcsa1, pVAX-hSMR3B, pVAX, or pVAX-hSlo) erectile function was determined by measuring the intracavernous pressure to blood pressure ratio (ICP/BP) with and without cavernous nerve (CN) stimulation, as previously described (7, 20, 36, 37). Briefly, to determine the intracorporal pressure response to stimulation of the CN, the rats were anesthetized with pentobarbital sodium (35 mg/kg ip). An incision was made in the perineum, and a window was made in the ischiocavernous muscle to expose the crura. The CN was identified on the posterolateral surface of the prostate gland. The isolated CN was directly electrostimulated with a delicate stainless steel bipolar hook electrode attached to a multijointed clamp. Each probe was 0.2 mm in diameter and the two poles were separated by 1 mm. Monophasic rectangular pulses were delivered by a signal generator (custom made and with built-in constant-current amplifier). The stimulation parameters were as follows: frequency, 20 Hz; pulse width, 0.22 ms; duration, 1 min; current, 0.75 and 4 mA. Changes in intracorporal pressure and systemic blood pressure (from the carotid artery pressure) were recorded at each intensity of stimulation. The mean ICP/BP and standard error of the mean (SE) were calculated for each treatment group. Significant differences between treatment groups were determined by Student’s t-test. Following determination of erectile function, corporal tissue was excised. It was either used immediately for histopathology or RNA analysis or stored at −80°C for protein analysis.

Detection of opiorphin peptides. One week following intracorporal injection of 100 μg pVAX-Vcsa1 or pVAX-hSMR3B the levels of opiorphin peptides were determined by radioimmunoassay (RIA). The antibodies against the sialorphin peptide and human homologue hSMR3 were custom made in rabbits (GenScript, Piscataway, NJ) against the peptides QHNPRY and TPGESQRGPRGPYP, respectively. The sialorphin peptide (QHNPRY) and hSMR3 peptide (TPGESQRGPRGPYP) were labeled by the method of Markwell et al. (19), using iodination beads. To 100 μl of phosphate-buffered saline, pH 7.4 (PBS), 1 mCi of carrier free Na125I (American Radiolabeled Chemicals, St. Louis, MO) and two iodobeads (Pierce, Rockford, IL) were added, incubated for 5 min at room temperature, followed by the addition of 50 μl of opiorphin peptide (100 μg) and incubated for a further 15 min at room temperature. The reaction was terminated by removing the beads. The radiolabeled opiorphin was separated on a Tris acryl GF-05 column (1 × 10 cm) preequilibrated with 4 column volumes of PBS followed by 2 column volumes of PBS containing 1% bovine serum albumin (BSA), and 1 column volume of PBS. Then 1-ml fractions were collected and the radioactivity (counts per minute) was determined using 10 μl of eluate from each fraction with a Perkin-Wallach Gamma counter. Based on the separation of the labeled peptide on a desalting column GF05, the peak fractions were pooled and the labeled peptide was stored at −20°C. The rat serum samples were collected in prechilled tubes containing 10 μl of protease inhibitor cocktail per milliliter of blood (Sigma). The samples were centrifuged immediately after collection at 7,000 g for 15 min at 4°C with a Sorvall centrifuge. The serum samples were aliquoted and stored at −80°C. Prior to the RIA 1 volume of serum samples were precipitated with 4 volumes of methanol containing 0.1% trifluoroacetic acid and the samples were homogenized with a Polytron minihomogenizer followed by centrifugation at 12,000 g for 20 min. The supernatant was separated and dried with a Savant SpeedVac centrifuge and stored at −20°C. The RIA was carried out by the method of Rougeot et al. (32), with known amount of standards and the serum samples in 100 μl of 0.2 M Tris·HCl pH 8.5 buffer containing 0.1% BSA and Triton X-100; 100 μl of opiorphin antibody (1:25 dilution) and 100 μl labeled peptide (15,000 cpm) were added and incubated at 4°C for overnight. Bound and free peptides were separated by addition of 1 ml of ice-cold propanol-immunoglobulin (normal rabbit serum, 10 μl). Samples were centrifuged and the radioactivity of the precipitate was determined by use of a gamma counter.

Isolation of RNA for microarray analysis. For the Gene Chip studies, the RG-U34A rat Gene Chip (Affymetrix, Sunnyvale, CA) containing ~8,799 genes was used. RNA was prepared from the corpora of animals 1 wk following intracorporal injection of 100 μg pVAX, pVAX-Vcsa1, pVAX-hSMR3B, or pVAX-hSlo. A bioanalyzer was used to determine that the isolated RNA was not degraded, and after confirmation of its quality the isolated RNA was reverse transcribed to cDNA, labeled with biotin, and fragmented according to Affymetrix protocols (http://www.affymetrix.com). Subsequent hybridization to microarray chips was done at the AECOM microarray core facilities by use of an Affymetrix Fluidics Station. Gene expression analysis was performed by using AffymetrixGUI software, (www.biocductor.org) as previously described (15, 38). Data were obtained from the following animals: five control corpora (intracorporally injected with 100 μg pVAX), three intracorporally injected with 100 μg pVAX-hSMR3B, four intracorporally injected with 100 μg pVAX-Vcsa1, and four intracorporally injected with 100 μg pVAX-hSlo. We took a very conservative analytical approach in this study to offset the relatively small sample size [as previously described (15)]; the major impact of the small sample size is expected to be that fewer gene changes are detected.

Isolation of RNA for quantitative RT-PCR. Total RNA was extracted from frozen tissue with TRIzol according to the manufacturer’s instructions as previously described (35, 36, 38). Briefly, ~100 mg tissue was added to 1 ml TRIzol reagent and homogenized using a Polytron homogenizer (Brinkman, Westbury, NY) for 30 s. The homogenized tissues were incubated for 5 min at room temperature followed by addition of 200 μl of chloroform. After mixing, the aqueous phase was separated by centrifugation (12,000 g for 15 min) at 4°C and then transferred to a clean tube. The RNA was precipitated from the aqueous phase by addition of isopropl alcohol and pelleted by centrifugation at 12,000 g for 15 min at 4°C, washed once with 75% ethanol, and again pelleted at 12,000 g for 15 min. The ethanol was aspirated and the RNA pellet dried and then dissolved in sterile water. Five micrograms of total RNA were reverse transcribed to first-strand cDNA and primed with oligo(dT) using the Superscript (Invitrogen, Carlsbad, CA) First-Strand synthesis system for real-time PCR. RNA was denatured for 5 min at 65°C and immediately cooled on ice. Then RNA was combined with the Superscript II RT, 40 units of RNaseOUT recombinant ribonuclease inhibitor, and RT reaction buffer. Next cDNA synthesis was performed for 50 min at 42°C. RT products were amplified by use of SYBR Green 2× PCR Master Mix (PE Applied Biosystems, Warrington, UK). Real-time quantitative PCR analysis was performed using the 7,300 real-time PCR system (Applied Biosystems, Foster City, CA). The primers (designed by the Primer Express Software) used in these experiments are shown in Table 1. The PCR reactions for all samples were performed in 96-well plates, with 1 μl (5 ng) cDNA, 100 nM each primer, and 12.5 μl of
Table 1. Rat and mouse primers used to determine gene expression

<table>
<thead>
<tr>
<th>Primers used to determine gene expression in rat</th>
<th>Forward primer</th>
<th>Reverse primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-Actin</td>
<td>5′-CTT CAA CAC CCC AGC CAT GT-3′</td>
<td>5′-AGT AAC CCT GGA TCA ATA ACC-3′</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5′-CCG AGG GCC CAC TAA AGG-3′</td>
<td>5′-GCA TCA AAG GTG GAA GAA TGG-3′</td>
</tr>
<tr>
<td>Glyceraldehyde-3-phosphate dehydrogenase (GAPDH)</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>Forward primer</td>
<td>5′-GGG TGA CCC CCT GCA TAT GT-3′</td>
<td>5′-GGA GAT TCC GCA TCA TAC-3′</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5′-GCA TTC CCA GTC GGC TCA TAC-3′</td>
<td>5′-GCA AGC ACC ACC AGC TGG-3′</td>
</tr>
<tr>
<td>Arginase-I</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>Forward primer</td>
<td>5′-CTT TGG CGC CCT TTT GCA TTA-3′</td>
<td>5′-CTG TTT TCC TGG TTC AGA GA-3′</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5′-GTC TCA ACC TGC CCT TGC TCA-3′</td>
<td>5′-CAT AGC GCT CTG GCA ATC AA-3′</td>
</tr>
<tr>
<td>Arginase-II</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>Forward primer</td>
<td>5′-GGT GGA GCA TCA GCC TGC TTA-3′</td>
<td>5′-CCA TCA AAG GTG GAA GAA TGG-3′</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5′-TCC GCA TCA TCC AGC TCA TAC-3′</td>
<td>5′-GGA GAT TCC GCA TCA TAC-3′</td>
</tr>
<tr>
<td>Arginine-1</td>
<td>Forward primer</td>
<td>Reverse primer</td>
</tr>
<tr>
<td>Forward primer</td>
<td>5′-AGT ACC GGT CCT GCA TAT GT-3′</td>
<td>5′-AGT ACC GGT CCT GCA TAT GT-3′</td>
</tr>
<tr>
<td>Reverse primer</td>
<td>5′-GCA CCT GCG ATT ACC ACC AGC TGG-3′</td>
<td>5′-GCA CCT GCG ATT ACC ACC AGC TGG-3′</td>
</tr>
</tbody>
</table>

SYBR Green in a 25-μl reaction volume. The cycling conditions were as follows: activation of SYBR Green DNA polymerase at 95°C for 10 min, 40 cycles of denaturation at 95°C for 15 s, annealing/extension at 60°C for 1 min. Results from real-time PCR were presented as threshold cycles normalized to that of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene according to the method previously described (35–37). The relative quantified value for each target gene is expressed as 2^(-ΔΔCt) (Ct and Cc are the mean threshold cycle differences after normalization to GAPDH). Expression of transcript was analyzed by the comparative crossing threshold (Ct) method (also known as the 2^-ΔΔCt method). GAPDH was used to normalize samples. Statistical analysis was carried out by Student’s t-test.

Analysis of protein extracts. One week following intracorporal injection of 100 μg pVAX or pVAX-Vcsa1, the expression of polyamines in corporal tissue was analyzed. All steps for protein isolation were performed by using a Polytron homogenizer in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM ethylenediaminetetraacetic acid, 30 mM sucrose, and 10 μl of protease inhibitor cocktail (Sigma). Samples were centrifuged at 15,000 g for 30 min. The pellet was discarded and the protein concentration in the supernatant was determined by using the Bio-Rad DC Protein Assay kit; 50 μg of proteins were separated by electrophoresis on Nu PAGE 10% Bis-Tris gels (Invitrogen) and then transferred to a nitrocellulose membrane (Immun-Blot PVDF Membrane, Bio-Rad Laboratories, Hercules, CA) by semidy electrophoretic transfer for 1 h. The membranes were blocked 1 h at room temperature with Blotto-Tween (5% nonfat dry milk-0.05% Tween-20) and incubated with ODC (1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA), PDE5A (1:500, BD-Transduction Laboratories, San Jose, CA), eNOS (1: 500, Sigma), spermidine acetyltansferase (1:1,000, Abcam, Cambridge, MA), arginine antibody (1:500, Abcam), and nitr tyresine (1:500, Alexis Biochemicals, San Diego, CA) antibodies at room temperature for 1 h. The bound antibodies were detected with labeled horseradish peroxidase-labeled anti-mouse or anti-rabbit secondary antibody (1:10,000) (Santa Cruz Biotechnology) for 1 h at room temperature. Enhanced chemiluminescence was performed with Pierce ECL Western Blotting Substrate (Pierce), and bands were quantified by densitometry using Quantity One software (Bio-Rad Laboratories).

Polyamine analysis. One week following intracorporal injection of 100 μg pVAX or pVAX-Vcsa1, the expression of polyamines in corporal tissue was analyzed. Polyamine analysis in corporal tissue was carried out by dansylation of polyamines, followed by HPLC separation and fluorescence detection. First corporal tissue was homogenized in 5% perchloric acid by using a Polytron homogenizer at 4°C. Homogenates were centrifuged at 6,000 g for 15 min. The tissue supernatants were stored at −80°C, until further use. The polyamine standards 1,4-diaminobutane, spermidine trihydrochloride, and spermine tetrahydrochloride (Sigma) were prepared in water. The dansylation reaction was carried out by the method of Bencsik et al. (1). Briefly, 100 μl of saturated sodium carbonate and 200 μl of dansyl chloride (5 mg/ml in acetone) were added to 100 μl of the tissue supernatant or the polyamine standards and incubated at 56°C for 1 h in the dark. Excess dansyl reagent was removed by adding 100 μl of freshly prepared L-proline (150 mg/ml in water) and incubated for half an hour at 56°C. The polyamines were extracted by using 500 μl toluene twice. The toluene extracts were dried by use of a Savant SpeedVac. The dried samples were dissolved in 100 μl of methanol and polyamines in the samples were analyzed by reverse-phase HPLC method using a Bondapak C18, 4.5 × 250 mm Waters column, and the eluted dansyl-labeled polyamines were detected with a Shimadzu fluorescence detector (12).

Histology. Histology was performed at the Albert Einstein College of Medicine Histology and Comparative Pathology Facility. Penes were fixed with 10% formalin overnight, then embedded in paraffin, sectioned at a thickness of 5 μm. Sections were stained on a Microm DS 50 slide stainer (Global Medical Instrumentation, Ramsey, MN) with hematoxylin and eosin (H&E). H&E sections were counterstained with Permount mounting medium (Fisher Scientific, Pittsburgh, PA). Additional sections were stained with Masson’s trichrome stain, and the measurement of collagen to smooth muscle ratio was assessed as described in Ref. 3.

RESULTS

Microarray analysis reveals that the ODC transcript is upregulated in animals with opioiphin-induced experimental priapism. One week following intracorporal injection of the plasmids expressing the opioiphins (100 μg pVAX-hSMR3B and pVAX-Vcsa1), circulating levels of the hSMR3B peptide product could be detected at ~2.5 ng/ml (Fig. 1A) and circu-
lating levels of sialorphin increased approximately twofold over endogenous levels (Fig. 1B). As previously described, intracorporal injection of both plasmids induced a priapic-like condition in the retired breeder rat corpora (35–37). Evidence for this was an increased basal ICP/BP (Fig. 1C) and visual and histological evidence of vasocongestion at necropsy (Fig. 1D). The empty vector backbone, 100 μg pVAX, was used as a control, and 100 μg pVAX-hSlo was used as a control for a plasmid that improves erectile function in the aging rat model without resulting in experimental priapism (20, 21).
Table 2. Genes most significantly changed in expression in response to intracorporal injection of 100 μg pVAX-Vcsa1, pVAX-hSMR3B, and pVAX-hSlo, compared with controls injected with 100 μg pVAX (as determined by microarray analysis)

<table>
<thead>
<tr>
<th>Affy ID</th>
<th>Gene</th>
<th>Fold Change</th>
<th>B-Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>J04792_at</td>
<td>ornithine decarboxylase 1</td>
<td>92.9</td>
<td>6.2</td>
</tr>
<tr>
<td>J04792_s_at</td>
<td>ornithine decarboxylase 1</td>
<td>15.8</td>
<td>5.0</td>
</tr>
<tr>
<td>rc_AA859899_at</td>
<td>serine proteinase inhibitor, clade F, member 1</td>
<td>6.4</td>
<td>3.4</td>
</tr>
<tr>
<td>rc_AA899552_at</td>
<td>aggrecan 1</td>
<td>6.0</td>
<td>3.2</td>
</tr>
<tr>
<td>X06107_r_at</td>
<td>insulin-like growth factor 1</td>
<td>2.7</td>
<td>3.0</td>
</tr>
<tr>
<td>rc_A8818593_at</td>
<td>development and differentiation enhancing factor 1</td>
<td>7.2</td>
<td>2.3</td>
</tr>
<tr>
<td>X05341_at</td>
<td>acetyl-coenzyme A-acyltransferase 2</td>
<td>0.3</td>
<td>2.1</td>
</tr>
<tr>
<td>rc_AA946503_at</td>
<td>lipocalin 2</td>
<td>32.0</td>
<td>1.9</td>
</tr>
<tr>
<td>U81037_at</td>
<td>neuron-glia-CAM related cell adhesion molecule</td>
<td>4.1</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Change in gene expression following intracorporal injection of pVAX-Vcsa1 compared with control (animals intracorporally injected with pVAX)

Change in gene expression following intracorporal injection of pVAX-hSMR3B compared with control (animals intracorporally injected with pVAX)

Change in gene expression following intracorporal injection of pVAX-hSlo compared with control (animals intracorporally injected with pVAX)

To gain insight into the molecular mechanisms associated with pVAX-Vcsa1 and pVAX-hSMR3B experimental priapism we compared gene expression (determined by microarray analysis using the Affymetrix RGU-34A chip) of animals following intracorporal injection of these plasmids compared with the control vector, pVAX. In addition, we determined the effect of 100 μg pVAX-hSlo on gene expression. The B-statistic was used to determine whether the changes in gene expression in the treated vs. control animals were significant. The B-statistic is the log odds of differential expression and takes into account the variability between replicate arrays. A B-statistic > 1 is considered significant. Overall, of the ~8,799 gene probes on the chips very few genes were observed to be significantly changed in expression in treated compared with control corporal tissue. In pVAX-Vcsa1, pVAX-hSMR3B, and pVAX-hSlo, 23, 4, and 65 gene probes were changed in expression compared with controls, respectively. The top 10 most significantly changed genes by microarray analysis are shown in Table 2. In the animals that were treated with pVAX-Vcsa1 and pVAX-hSMR3B one of the most significantly upregulated genes was ODC. Treatment with pVAX-Vcsa1 and pVAX-hSMR3B resulted in the upregulation of ODC expression by at least 15-fold. (ODC is represented by two sets of oligonucleotide probe sets on the RGU-34A chip, J04792_at and J04792_s_at). In the pVAX-hSlo-treated animals ODC was also upregulated (>5.5-fold), but by a significantly lower extent than in the pVAX-Vcsa1 and pVAX-hSMR3B-treated animals. Several other genes were changed in expression to the pVAX-Vcsa1 and pVAX-hSMR3B-treated animals, and several of these genes were common between the two groups, such as a serine proteinase inhibitor F1, insulin-like growth factor, and development and differentiation enhancing factor 1. However, because it has been previously reported that arginine metabolism can play a role in erectile physiology in animal models (2, 13, 24) we focused on a potential role of ODC and polyamine synthesis in the development of the priapic-like condition caused by overexpression of opiorphins.

Quantitative RT-PCR of genes involved in arginine metabolism and polyamine synthesis. We used quantitative RT-PCR to determine whether other genes in the ODC-polyamine synthetic pathway were changed in another set of animals treated with 100 μg pVAX-Vcsa1 or 100 μg pVAX-hSlo compared with animals treated with the empty vector (pVAX). Again we observed a significant sixfold upregulation of ODC expression in the pVAX-Vcsa1-treated animals (and to a lower extent, twofold, in the pVAX-hSlo-treated animals) (Table 3). Interestingly, arginase-I and -II, which are involved in the conversion of arginine to citrulline, were also upregulated, particularly in the pVAX-Vcsa1-treated animals (5.5-fold), but by a significantly lower extent than in the pVAX-Vcsa1 and pVAX-hSMR3B-treated animals. Several other genes were changed in expression to the pVAX-Vcsa1 and pVAX-hSMR3B-treated animals, and several of these genes were common between the two groups, such as a serine proteinase inhibitor F1, insulin-like growth factor, and development and differentiation enhancing factor 1. However, because it has been previously reported that arginine metabolism can play a role in erectile physiology in animal models (2, 13, 24) we focused on a potential role of ODC and polyamine synthesis in the development of the priapic-like condition caused by overexpression of opiorphins.
sion of arginine to ornithine (the substrate for ODC), were observed to be >16-fold and >5-fold upregulated, respectively. Arginase-I and -II are both present on the RGU-34A microarray chip; however, although they were detected as upregulated, the conservative analysis we performed did not detect their expression as significantly changed. We also determined the expression of genes downstream of the metabolism of ornithine that are involved in polyamine synthesis (polyamine oxidase, spermidine acetyltransferase, S-adenosylmethionine decarboxylase, spermine synthase, and spermidine synthase). With the exception of spermine synthase, all the genes investigated involved in the polyamine synthetic pathway were significantly upregulated in pVAX-Vcsa1-treated animals compared with controls (Table 3). In animals treated with pVAX-hSlo (which does not cause experimental priapism) there was no significant upregulation of the other genes involved in polyamine metabolism that were investigated. It is apparent from Tables 2 and 3 that the priapic-like condition induced by intracorporal injection of pVAX-Vcsa1 is accompanied by upregulation of genes involved in arginine metabolism and polyamine synthesis.

Polyamines are overexpressed in animals with priapism. The upregulation of genes involved in polyamine synthesis would be expected to result in accumulation of polyamines in the corporal tissue. Therefore, we compared the levels of polyamines (putrescine, spermidine, and spermine) in the corpora of pVAX-Vcsa1-treated animals with control animals treated with the control plasmid (pVAX). Dansylated polyamines were separated by HPLC and quantified by comparing fluorescent intensity with standards. The results are shown in Fig. 2. The level of the polyamine putrescine was increased by 12.5-fold in pVAX-Vcsa1 gene-treated corpora tissues compared with control pVAX tissues. The levels of spermidine and spermine were not significantly different between animals treated with pVAX or pVAX-Vcsa1.

Expression of proteins involved in arginine metabolism and involved in priapism. We investigated the change in protein expression of key enzymes involved in arginine metabolism in the corpora of pVAX-Vcsa1-treated animals to pVAX control-treated animals. In addition we also investigated the expression of other proteins previously reported as playing a role in priapism (6). Equal loading of proteins was confirmed using the housekeeping gene GAPDH. As shown by the representative Western blots and densitometric analysis (Fig. 3), the expression of arginase-I and -II and ODC correlated with the increase in the gene expression determined by quantitative RT-PCR (Table 3). A key enzyme in polyamine synthesis (spermidine acetyltransferase) was also confirmed to be significantly upregulated. In addition, two enzymes that have been shown to play a role in priapism (endothelial nitric oxide synthase and PDE5) were shown to be significantly downregulated in animals treated with pVAX-Vcsa1.

We were unable to detect any significant differences in the background levels of NO production between tissue extracts from pVAX- and pVAX-Vcsa1-treated animals. However, we determined the levels of proteins with nitrotyrosine residues in animals treated with pVAX and pVAX-Vcsa1. Nitrosylation of proteins results from reaction of NO to generate peroxynitrite radicals. A typical result is shown in Fig. 4. Densitometric analysis of blots from six animals showed that animals treated with pVAX-Vcsa1 had significantly lowered levels of nitrotyrosine proteins (0.6 ± 0.4) compared with animals treated with pVAX alone.

**ODC inhibitors prevent experimental priapism caused by intracorporal injection of plasmids expressing opiorphins.** The observation that ODC was significantly upregulated in animals with Vcsa1-induced experimental priapism could either suggest a direct involvement of the enzyme in priapism or an

<table>
<thead>
<tr>
<th>Fold Change in ODC</th>
<th>Fold Change in Spermidine Synthase</th>
<th>Fold Change in Spermine Synthase</th>
<th>Fold Change in Putrescine</th>
<th>Fold Change in Spermidine</th>
<th>Fold Change in Spermine</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVAX-Vcsa1</td>
<td>1.0 ± 0.5</td>
<td>0.7 ± 0.4</td>
<td>0.2 ± 0.1</td>
<td>1.0 ± 0.5</td>
<td>0.05 ± 0.1</td>
</tr>
<tr>
<td>pVAX-hSlo</td>
<td>1.0 ± 0.6</td>
<td>0.08 ± 0.06</td>
<td>0.18 ± 0.06</td>
<td>0.35 ± 0.5</td>
<td>0.14 ± 0.05</td>
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</tbody>
</table>

Values are means ± SD. GAPDH was used for the normalization of gene expression. Five animals were used in each treatment group, and quantitative RT-PCR was performed in triplicate for each animal. ODC, ornithine decarboxylase.
indirect response to priapism, for example through priapic-induced tissue damage. To discern these two possibilities we determined whether a well-characterized inhibitor of ODC [1,3-diaminopropane (33)] could prevent the effect of 100 μg pVAX-Vcsa1 (or a plasmid carrying the human homologue, pVAX-SMR3B) in causing experimental priapism in the rat model. We compared the effect of intracorporal injection of pVAX-Vcsa1 (or the human homologue SMR3B) on erectile physiology in animals with or without the presence of 1,3-diaminopropane in their drinking water. As shown in Fig. 2, the level of the polyamine putrescine (detected by HPLC analysis) was significantly reduced in the pVAX-Vcsa1 animals receiving 1,3-diaminopropane in their drinking water for 1 wk compared with animals treated with pVAX-Vcsa1, but without the ODC inhibitor present in their drinking water.

One week following treatment with plasmids expressing the opiorphin encoding genes, following necropsy, seven of ten animals displayed visual evidence of a priapic-like condition (Table 4) similar to our previously reported observations of the effect of pVAX-Vcsa1 or pVAX-hSMR3B (35–37). Histological and pathological examination of these seven animals demonstrated that they had increased vasocongestion in the corpora cavernosa. Vasocongestion, which is a result of increased vascular blood flow, would be an expected consequence of a priapic-like condition (see Fig. 1D). In addition, the basal (no stimulation of the cavernous nerve) ICP/BP ratio is significantly elevated in animals treated with plasmids expressing the opiorphin gene, suggesting an elevated influx of blood into the corpora, which might also be expected in a priapic-like episode (Figs. 1C and 5A). However, when animals were intracorporally injected with plasmids expressing the opiorphin-encoding genes (pVAX-Vcsa1 or pVAX-hSMR3B) but given the ODC inhibitor 1,3-diaminopropane in their drinking water they did not develop a priapic-like condition (Table 4). In addition, their baseline ICP/BP was not

Table 4. Visual assessment of erectile function following intracorporal gene transfer of 100 μg the empty vector plasmid or plasmids expressing opiorphin homologues (Vcsa1 or hSMR3B) in the presence or absence of ODC inhibitor

<table>
<thead>
<tr>
<th>Animal</th>
<th>0.75 mA</th>
<th>4 mA</th>
</tr>
</thead>
<tbody>
<tr>
<td>N Pr NE PE FE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pVAX</td>
<td>5 0</td>
<td>5 0</td>
</tr>
<tr>
<td>pVAX-Vcsa1</td>
<td>5 4</td>
<td>1 4</td>
</tr>
<tr>
<td>pVAX-Vcsa1 (ODC inhibitor)</td>
<td>5 0</td>
<td>0 5</td>
</tr>
<tr>
<td>pVAX-hSMR3B</td>
<td>5 3</td>
<td>0 5</td>
</tr>
<tr>
<td>pVAX-hSMR3B (ODC inhibitor)</td>
<td>5 0</td>
<td>2 2</td>
</tr>
</tbody>
</table>

Pr, visual evidence of priapism; NE, no erection; PE, partial erection; FE, full erection.
In sickle cell mice, priapism is accompanied by changes in the expression of key enzymes in polyamine synthesis and mouse opiorphins. To determine whether similar changes in the polyamine synthetic pathways occurred in another model of priapism we compared changes in gene and protein expression in the corpora of sickle cell mice (27) to age-matched controls of C57BL/6, the parent mouse strain. Three life stages of the mice were studied. 1) Prepriapic mice (∼5 wk of age): sickle cell mice were not observed to have any visual evidence of priapism. Histopathology of the corpora of these mice showed no evidence of vasocongestion (Fig. 6A, left). 2) Priapic mice (∼10–12 wk of age): mice were visually observed to have priapism (a prolonged erection without sexual stimulation). Histopathology of these mice showed evidence of vasocongestion but presented no evidence of necrosis of the corporal tissue (Fig. 6A, middle). 3) Postpriapic mice (>12 wk of age): mice at necropsy had visible signs of necrosis of corporal tissue. Histopathology of the corporal tissue from these animals had more dilated spaces with vasocongestion as well as evidence of periprostatic inflammation and mild edema associated with neutrophilia (Fig. 6A, right). Trichrome staining of postpriapic mouse corporal sections showed a significant increase in the fibrosis scale (the ratio of collagen to smooth muscle) compared with the prepriapic or priapic mice (Fig. 6B). Age-matched control animals presented no evidence of vasocongestion or inflammation.

We investigated the expression of two mouse genes related to Vcsa1 (mSMR2, acc. no. NM_021289 and mSMR3a, acc. no. NM_011422) (39). Of these two genes only mSMR2 potentially encodes a pentapeptide (QRGFR) with homology to sia-lorpin (QHNPR). mSMR2 was significantly upregulated (twofold) in sickle cell mice at a life stage when there is no evidence of priapism (5 wk, Fig. 6B) relative to the age-matched control mice prior. At a life stage when sickle cell animals develop priapism there was an approximately eightfold increase in expression of the mSMR2 gene in the sickle cell mice compared with controls. In contrast, there is no significant change in the expression levels of mSMR3a at any of the life stages of the sickle cell mice investigated. As priapism developed and progressed to obvious signs of necrosis of the corporal tissue, key enzymes of polyamine synthesis (arginase-I and -II and ODC) were also significantly upregulated at the level of transcription (Fig. 6C). We confirmed the upregulation of key enzymes in the polyamine synthetic pathway (arginase-I and -II and ODC) at the protein level in the same set of animals (Fig. 6D). Equal loading of proteins was confirmed by use of the housekeeping gene GAPDH.

**DISCUSSION**

We have previously shown that intracorporal injection of retired breeder rats with plasmids expressing opiorphins induces a priapic-like condition in rats (35–37). In the present paper we demonstrate by microarray analysis that intracorporal injection of plasmids expressing opiorphin genes (pVAX-SMR3B or pVAX-Vcsa1) results in significant upregulation of the gene for ODC compared with control animals treated with the vector backbone (pVAX). Intracorporal injection of animals with pVAX-hSlo, which has been demonstrated to improve erectile function (20, 21), but not to induce experimental priapism, had significantly lower increases in ODC expression. We confirmed by quantitative RT-PCR in animals treated with pVAX-Vcsa1 that not only was ODC upregulated, but also arginase and several other genes involved in polyamine synthesis. Western blot analysis confirmed the upregulated expression of two enzymes in the polyamine synthetic pathway (ODC and spermidine acetyltransferase). In addition, the polyamine putrescine was detectable at elevated levels in the pVAX-Vcsa1-treated animals compared with controls. The elevation of enzymes in the polyamine synthetic pathway could directly
result in the priapic-like condition or be a response to the condition, for example, through the Vcsa1- or hSMR3B-induced priapic-like condition resulting in corporal tissue damage. However, we demonstrate that a well-characterized inhibitor of ODC [1,3-diaminopropane (33)] can inhibit the polyamine synthetic pathway and prevent an opiorphin-induced priapic-like condition. This observation suggests that the elevated levels of ODC play a direct role in the development of the experimental priapism. It remains to be established whether other enzymes in the polyamine pathway, such as arginase, play a direct role in the development of the priapic-like condition. We also demonstrate in another model of priapism, the sickle cell mouse, that prior to a life stage when there is evidence of priapic-like episodes there is upregulation of the mouse opiorphin homologue mSMR2. At a life stage when there is priapism, mSMR2 is further elevated, accompanied by the upregulation of key enzymes involved in polyamine synthesis.

Fig. 6. A: histopathology demonstrating the difference between the different life stages of the sickle cell mice. Left: pre-priapic mouse (Pre-P). There was no evidence of priapism in animals, and histopathology showed no evidence of vascongestion. Middle: priapic mouse (P). Animals were observed to have priapism, and histopathology demonstrates vasocongestion of the corporal tissue. Right: post-priapic mouse (Post-P). Animals had obvious signs of necrosis of the corporal tissue. Histopathology demonstrated evidence of enlarged spaces with vascongestion and inflammation of corporal tissue. Sections of each panel are enlarged, and the presence of blood cells within the vascular spaces enhanced to highlight vascongestion. B: representative trichrome staining of penis sections from pre-priapic, priapic, and post-priapic sickle cell mice (bottom). Bar graph demonstrating the ratio of collagen to smooth muscle (SM) in the different groups (N = 3 animals per group) (top). The post-priapic animals had a significant increase in the collagen to smooth muscle ratio compared with the pre-priapic and priapic mice (P < 0.05). C: quantitative RT-PCR was performed on corporal tissue harvested from the sickle cell mice at the life stages described above and compared with age-matched controls [the parent strain of the sickle cell mice (C57BL/6)]. Quantitative PCR was performed by pooling RNA from 2 animals, in duplicate from each point, and performing quantitative RT-PCR in triplicate. Bars represent the fold change in expression of the gene in sickle cell mice compared with age-matched control (C57BL/6) determined from 6 quantitative RT-PCR reactions for each gene, normalized to the housekeeping gene (GAPDH). Error bars represent SD. *Significantly changed gene expression from control. D: representative Western blot for protein expression in pooled samples from the corpora of 2 control (C57BL/6) or 2 sickle cell animals. Protein loading was normalized by using the Bio-Rad protein assay prior to loading and confirmed by GAPDH detection after transfer to PVDF membranes. The fold change in expression and SD of the protein in sickle cell mice compared with age-matched controls was determined by densitometric analysis of at least 2 gels for each of 2 pooled samples for each group, normalized to GAPDH. *Significantly changed protein expression compared with control.
The mechanism by which the opiorphins cause experimental priapism in animals may be through their ability to act as NEP inhibitors and thereby affect peptide signaling mediated through GPCR. When the expression of Vcsa1 was knocked down in rat corporal smooth cells, microarray analysis revealed that as an ontological group GPCR were upregulated (38). The effect by opiorphins on GPCR activity and expression would be expected to change intracellular cAMP and cGMP levels, which have been demonstrated to be involved in development of the priapic-like condition in Ada−/− mice. The overexpression of opiorphins may also result in changes at the biochemical level similar to those experienced in the eNOS−/− knockout mice where there was observed to be a reduction in PDE5 expression (5, 6). We demonstrate that animals treated with plasmids expressing the opiorphins also had decreased PDE5 and eNOS expression (Fig. 3). Studies by Burnett et al. (5) and Champion et al. (6) suggested that following sexual stimulation (that results in increased release of neuronal NO), large amounts of cGMP are produced, and because of insufficient amounts of functional PDE5 being present to degrade the cyclic nucleotide, this results in excessive erectile tissue relaxation. In rats intracorporally injected with plasmids expressing opiorphins the resultant upregulation of ODC and the polyamine pathway may channel arginine away from nitric oxide synthase, causing reduced expression of NO, similar to the eNOS−/− mice (see Fig. 7). Compounding this effect may be a direct effect of increased polyamine levels in corporal tissue. Polyamines have been shown to relax bladder and corporal smooth muscle tissues in vitro (Ref. 25 and data not shown). Decreased tone of corporal smooth muscle tissue would enhance blood flow into corporal tissue, potentially resulting in vasocongestion and the ensuing priapic-like condition seen following intracorporeal injection of plasmids expressing the opiorphins. Following stimulation of the cavernous nerve the erectile response shows no significant difference between the animals treated with pVAX-Vcsa1/hSMR3B and those treated with pVAX-Vcsa1/hSMR3B and an ODC inhibitor (Fig. 5). This effect may be explained by the erectile response to stimulation (measured by ICP/BP) being nonlinear. There is a limit to the maximum ICP/BP ratio, causing a plateauing of response to stimulation above and ICP/BP of 0.7. Differences in ICP/BP between groups can therefore get masked at higher levels of stimulation.

The mechanism by which the opiorphins result in increased ODC expression is also likely to be mediated through their action as NEP inhibitors modulating the activation of GPCR by peptide agonists (31, 40). Several reviews have highlighted that ODC and other members of the polyamine synthetic pathway are regulated through GPCR signaling pathways (28, 29). Therefore, the upregulation of ODC observed in pVAX-Vcsa1-treated animals may be mediated through the opiorphins changing the expression or activity of GPCR involved in the regulation of enzymes involved in the polyamine synthetic pathway.

It has previously been suggested that the regulation of NO and polyamine metabolic pathway plays a critical role in erectile function (2, 13, 24). However, our report is the first to suggest that an overactivation of the polyamine pathway results in priapism. Presumably, the improved erectile function that we have previously reported, following intracorporal injection of lower doses of plasmids expressing opiorphins (35, 36), represents a condition in which there is increased relaxation of the corporal smooth muscle tissue resulting in improved erectile function, without the excessive accumulation of blood in the intracavernosal spaces that would result in the priapic-like condition.

We also demonstrate that in sickle cell mice, a well-documented model of priapism, at a life stage when there is no evidence of priapism, there is already significant (twofold) upregulation of the mouse opiorphin homologue, mSMR2. This increases to eightfold at a life stage when the mice exhibit priapism, accompanied by an increase in expression of key enzymes in the polyamine synthetic pathway. Upregulation of opiorphin genes and their products in human patients with sickle cell disease may therefore act as biomarkers to determine which patients are at risk of developing priapism. We and others have documented that Vcsa1 expression is androgen regulated (8, 30). It is possible that changes in expression of the opiorphins as a result of androgen levels (for example, occurring with the onset of puberty or androgen supplementation) may increase the probability of priapism through activation of polyamine synthesis.

There is strong evidence that the rat and human homologues of opiorphins perform similar functions in erectile physiology. For example, intracorporal injection of both the rat and human genes encoding opiorphins (pVAX-Vcsa1 and pVAX-hSMR3B reported here, and pVAX-ProL1 reported in Ref. 36) causes the priapic-like condition in retired breeder rats. Several experiments presented in this paper suggest that pVAX-Vcsa1 and pVAX-hSMR3B cause similar changes in the expression of genes involved in polyamine synthesis. The homology of the opiorphin peptides from rat and human and their similar effect on an animal model suggest that the molecular mechanisms regulated by Vcsa1 in the rat may be similar to ProL1 and hSMR3B in humans. Indeed, pathological states in patients resulting in erectile dysfunction have been shown to be associated with downregulated expression of ProL1 and hSMR3B (36, 37).

The severe negative impact of priapism on the health of patients has spurred new research into the development of pharmacological interventions for its treatment. Recently, the use of PDE5 inhibitors in its treatment has been advocated (4).
However, it is important to explore and evaluate new management strategies for the disorder. Arginine supplementation has been identified as improving microvascular function that is associated with several pathologies (14, 17, 18, 24, 26, 34). The identification that inhibiting arginine catabolism through the use of ODC inhibitors can prevent the development of a priapic-like condition in at least one animal model identifies a new potential treatment paradigm that could be applied to patients with priapism.

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REFERENCES


