Reversal of diabetic vasculopathy in a rat model of type 1 diabetes by opiorphin-related peptides

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Departments of 1Urology and 2Physiology and Biophysics, 3Dominic P. Purpura Department of Neuroscience, Albert Einstein College of Medicine, Bronx, New York; and 4Institut Pasteur, Unité de Biochimie Structurale et Cellulaire, Paris, France

Submitted 15 April 2011; accepted in final form 16 July 2011

Calenda G, Tong Y, Kanika ND, Tar MT, Suadicani SO, Zhang X, Melman A, Rougeot C, Davies KP. Reversal of diabetic vasculopathy in a rat model of type 1 diabetes by opiorphin-related peptides. Am J Physiol Heart Circ Physiol 301: H1353–H1359, 2011. First published July 22, 2011; doi:10.1152/ajpheart.00383.2011.—Diabetes results in a myriad of vascular complications, often referred to as diabetic vasculopathy, which encompasses both microvascular [e.g., erectile dysfunction (ED), retinopathy, neuropathy, and nephropathy] and macrovascular complications (hypertension, coronary heart disease, and myocardial infarction). In diabetic animals and patients with ED, there is decreased opiorphin or opiorphin-related gene expression in corporal tissue. Both opiorphin and the rat homologous peptide sialorphin are found circulating in the plasma. In the present study, we investigated if diabetes induced changes in plasma sialorphin levels and if changes in these levels could modulate the biochemistry and physiology of vascular smooth muscle. We show that circulating sialorphin levels are reduced in a rat model of type 1 diabetes. Intracorporeal injection of plasmids expressing sialorphin into diabetic rats restores sialorphin levels to those seen in the blood of nondiabetic animals and results in both improved erectile function and blood pressure. Sialorphin modulated the ability of C-type natriuretic peptide to relax both corporal and aortic smooth muscle strips and of bradykinin to regulate intracellular calcium levels in both corporal and aortic smooth muscle cells. We have previously shown that expression of genes encoding opiorphins is increased when erectile function is improved. Our findings thus suggest that by affecting circulating levels of opiorphin-related peptides, proper erectile function is not only an indicator but also a modulator of overall vascular health of a man.

hypertension; erectile dysfunction; sialorphin

IT IS ESTIMATED THAT MORE than 23 million people in the USA, 7.8 percent of the population, suffer from diabetes (http://diabetes.niddk.nih.gov/DM/PUBS/statistics/). Diabetes reduces life expectancy by 5–10 yr with premature cardiovascular disease (CVD) being the commonest cause of morbidity and mortality (33). There are a myriad of vascular complications resulting from diabetes, often referred to as diabetic vasculopathy, which encompasses both microvascular [e.g., erectile dysfunction (ED), retinopathy, neuropathy, and nephropathy] and macrovascular complications (hypertension, coronary heart disease, and myocardial infarction) (9, 31). Recently, it has been recognized that ED is a strong predictor of CVD although the pathophysiological mechanism is unclear. Nevertheless, the American Medi-cal Association now recommends that patients with ED should be investigated for signs of CVD (2, 14, 15, 19, 39, 41).

A major contributing factor to the development of vascular complications is changes in the physiology of the smooth muscle tissue that result in increased tonicity. It is becoming apparent that correcting the underlying smooth muscle dysfunction can be effective in treating multiple diseases associated with smooth muscle pathology. For example, phosphodi-esterase type-5 inhibitors (such as sildenafil), which are effective treatments of ED because of their ability to induce relaxation of corporal smooth muscle (CSM) tissue, are now being prescribed for the treatment of primary pulmonary hypertension (32). Although there are several reports suggesting that smooth muscle dysfunction share common mechanisms of development (12, 18, 29), the precise mechanisms contributing to increased smooth muscle tone as a consequence of diabetes remain to be elucidated. We recently demonstrated that members of the opiorphin family of peptides play an important role in regulating CSM tone, thereby affecting erectile physiology. We (10, 42–44) have also shown that the opiorphin family members can act as markers for erectile function in both patients and animal models of diabetes. Furthermore, we (42) have reported that expression of the Vcsal1 gene encoding the rat homologue of human opiorphin (called sialorphin) is decreased in CSM tissue in animal models of diabetes.

Human opiorphin and rat sialorphin have recently been shown to act as potent endogenous inhibitors of neutral endopeptidase (NEP) and aminopeptidase-N (AP-N; Refs. 35, 48). Their biochemical mechanism of action is therefore likely to involve protection from inactivation by NEP and/or AP-N of endogenous signal peptides released in response to threat-inducing stimuli. The predominant sites of Vcsal1 expression in the rat are the submandibular gland, the prostate gland, and the CSM tissue. The Vcsal1 gene encoding a 17-kDa precursor protein is processed into the sialorphin pentapeptide, which is released into the blood stream. In humans, there are three homologous genes, ProL1, hSMR3A, and hSMR3B (43). The name opiorphin corresponds to the mature pentapeptide product of the ProL1 precursor protein (48). The other human gene homologue, hSMR3, is derived from two closely related genes (hSMR3A and hSMR3B), both of which could be posttranslationally processed to the same mature hSMR3-pentapeptide.

NEP plays a role in regulating the tone of smooth muscle. Sialorphin has been shown to enhance relaxation of CSM tissue by C-type natriuretic peptide (CNP; Ref. 10) and synthetic NEP inhibitors have been demonstrated to relax vascular smooth muscle tissue when used in combination with CNP (21). In addition, coadministration of thiorphan (a synthetic specific NEP inhibitor) has been suggested to increase efficacy
of angiotensin-converting enzyme (ACE) inhibitors in lowering blood pressure (BP) and to improve hemodynamic measurements following heart failure (5, 13, 24). Another NEP inhibitor drug, omapatrilat, also initially showed promise as an antihypertensive agent. However, because of safety concerns it was withdrawn from clinical trials in 2003 (47).

The reports describing shared mechanisms for the development of ED and vascular smooth muscle pathologies led us to investigate if the effect of opiorphin-related peptides on the physiology and biochemistry of CSM tissue and erectile function could be transposed to other vascular/smooth muscle systems. We investigated if restoring the circulating levels of sialorphin in diabetic rats could reduce the effects of diabetes on the vascular system. We also determined if sialorphin exerts similar biochemical effects on vascular and CSM cells. Overall our findings suggest that sialorphin and other opiorphin-related peptides might play a role as a general regulator of smooth muscle physiology.

MATERIALS AND METHODS

Diabetic rats. Forty-one F-344 rats (Taconic Farms, Germantown, NY) aged 8–10 wk (200–240 g) were used in these studies. The number of replicates in each experiment is given in the Fig. legends. Rats were fed Purina laboratory rodent chow ad libitum and housed individually with a 0700–1900 light cycle. Rats were made diabetic by a single intraperitoneal injection of streptozotocin (STZ; 35 mg/kg) dissolved in citrate buffer (60 ml of 0.1 mol/l citric acid and 40 ml of 0.2 mol/l Na2HPO4, pH 4.6), as previously described (17). Age-matched control (AMC) animals received an injection of vehicle only. Eight weeks after diabetes induction, one group of diabetic animals was treated daily with 2 U insulin subcutaneously (Eli Lilly) for 1 wk. Diabetic rats.

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Rat CSM cells. Rats were euthanized by CO2 inhalation, the corporal tissue was harvested, and CSM cells were isolated as previously described (3). Briefly, corporal tissue was enzymatically dissociated by incubating with 30 U/ml papain and 1 mg/ml dithioerythritol followed by incubation in 125 U/ml collagenase and 2.5 U/ml elastase at 37°C in a solution containing the following: 137 mM NaCl, 5.6 mM KCl, 1 mM MgCl2, 0.42 mM Na2HPO4, 0.44 mM NaH2PO4, 4.2 mM NaHCO3, 10 mM HEPES, and 1 mg/ml albumin at 37°C (physiological salt solution buffer). After enzymatic dissociation, the cells were thoroughly washed by centrifugation twice in physiological salt solution buffer to remove residual enzymes. Dissociated CSM cells were then plated in cell culture flasks and grown in 1.0 g/l glucose DMEM media (GIBCO, Invitrogen, Carlsbad, CA) supplemented with 10% FBS (GIBCO) and 1% penicillin-streptomycin (Invitrogen) and cultured for no more than three passages.

Aortic smooth muscle cells. The A7r5 aorta-derived smooth muscle cell line was obtained from ATCC (Manassas, VA) and cultured in DMEM media (GIBCO) supplemented with 10% fetal FBS (GIBCO, Invitrogen) and 1% penicillin-streptomycin (Invitrogen) following ATCC guidelines for no more than three passages.

Sialorphin injection and gene transfer. Intracorporal injection of 80 μg plasmids for expression of opiorphin homologue genes (pVAX-Vcsa1 or pVAX-hSMR3B), or pVAX, or pVAX-hSlo gene was previously described (16). 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. One-hundred micrograms of sialorphin (>98% purity; Sigma, St. Louis, MO) were intracorporally injected in 150-μl carrier as previously described (10).

Quantitative RT-PCR. Quantitative RT-PCR was performed essentially as described in (42). Briefly, total RNA was extracted from frozen tissue with TRIzol according to the manufacturer’s instruction. Total RNA was reverse-transcribed to first-strand cDNA followed by amplification using Sybr Green 2X PCR Master Mix (PE Applied Biosystems, Warrington, UK). Real-time quantitative PCR analysis was performed using the 7300 real-time PCR system (Applied Biosystems, Foster City, CA). The primers for Vcsa1 were as follows: forward primer, 5’-GAGGTTGTCAGAGGCCC-3’; and reverse primer, 5’-GACAGTGAGCTGGACACATCTAGA-T3’ (nucleotides 147–163 and 364–384, respectively). GAPDH (forward primer, 5’-GC-CGCTGTCCACACCCTC-3’; and reverse primer, 5’-GCTGT-GCTTGCCTGTTCTAC-3’) was used as an endogenous control. The expression of transcripts was analysed by the comparative crossing threshold (Ct) method [also known as the 2ΔΔCt method; Ref. 20]. This method was applicable because the efficiency of the primers in generating Vcsa1 product was comparable to that of the GAPDH gene.

Detection of sialorphin peptide. One week following intracorporal injection of either 80 or 100 μg pVAX-Vcsa1 (as described in RESULTS) the sialorphin peptide was determined by RIA as previously described (16, 36). The limit of detection of sialorphin was ~100 fmol of a standard peptide with an interassay variation coefficient of ~10% (n = 10).

Measurement of BP and intracorporal pressure. The method for determining systemic BP and intracorporal pressure (ICP) has been previously described (8, 16). Briefly, animals were first anesthetized by pentobarbital sodium (35 mg/kg ip). To monitor ICP, a 23-gauge cannula connected to a pressure line was inserted into the right corpus cavernosum and to determine BP a 25-gauge cannula connected to a pressure line was placed into the carotid artery. Both BP and ICP pressure lines were connected to a pressure transducer that was in turn connected via a transducer amplifier (ETH 400; CB Sciences) to a data acquisition board that allows a real-time display and recording of pressure measurements. The pressure transducers and analog-to-digital board were calibrated in centimeters of H2O. The cavernous nerve was identified posterolateral to the prostate on both sides, and the stainless steel bipolar wire electrodes were placed around these structures for electrical stimulation at 0.75 and 4 mA for ~1 min. Changes in ICP and BP were recorded at each level of neurostimulation. Results were expressed as the means ± SE.

Determination of muscle strip tone. Four longitudinal strips of cavernosal tissue or aortic rings were suspended between small stainless steel wire hooks, and muscle strips were attached to the pressure transducer. One end of the muscle strip was connected to a H-shaped brass holder that contacted the metal electrode. The other end of the muscle strip was connected to the pressure transducer. The longitudinal strips were preconditioned by electrical stimulation before each experiment.

Table 1. Expression levels of Vcsa1 gene in various tissues in 8-wk STZ-diabetic animals and age-matched controls

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Relative Vcsa1 Expression to Calibrator [Vcsa1 Expression in SMG of Age-Matched Control (non diabetic) Animals]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Submandibular gland</td>
<td>0.32 (0.017)</td>
</tr>
<tr>
<td>2. Corporal tissue</td>
<td>0.30 (0.007)</td>
</tr>
<tr>
<td>3. Prostate</td>
<td>0.003 (0.002)</td>
</tr>
<tr>
<td>4. Testis</td>
<td>0.00009 (0.00008)</td>
</tr>
<tr>
<td>5. Hypothalamus</td>
<td>0.0032 (0.0003)</td>
</tr>
<tr>
<td>6. Pituitary gland</td>
<td>0.00009 (0.00008)</td>
</tr>
<tr>
<td>7. Aorta</td>
<td>0.0012 (0.0003)</td>
</tr>
<tr>
<td>8. Liver</td>
<td>0.00009 (0.00008)</td>
</tr>
</tbody>
</table>

For age-matched controls, the submandibular gland (SMG) was used as calibrator tissue. STZ, streptozotocin.
surgical hooks in a tension-measuring device (Multimyograph Model 610M; Copenhagen, Denmark) that allows simultaneous monitoring of four muscle strips. Tissue was equilibrated for 90 min in 6 ml Krebs-Henseleit buffer (in mM: 110 NaCl, 4.8 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, 11 glucose, and dextrose in glass-distilled water). Organ chambers were maintained at 37°C and continuously bubbled with 95% O₂-5% CO₂ to maintain a mean pH of 7.4 ± 0.1. Tension developed by the muscle strips was continuously recorded using Powerlab software (Chart version 4.2.4; AD Instruments, CO) on a dedicated computer. The smooth muscle strips were first precontracted with phenylephrine (10⁻⁶ M). Then, relaxation was induced using CNP dissolved in the carrier DMSO (final concentration in organ bath, 10⁻⁶ M CNP, 0.1% DMSO). The change in the rate of relaxation after the addition of 1 μg/ml sialorphin was derived from the slope of the recording, as the change in tension (g) over time (min). The results from four separate strips (performed in duplicate) from four rats were averaged, and the significance was determined using Student’s t-test.

Intracellular calcium transients. Because intracellular Ca²⁺ concentrations play an important role in the regulation of smooth muscle tone, and thereby physiology, the ability of sialorphin to effect changes in cytosolic Ca²⁺ levels was measured as previously described by us (3, 40). Briefly, cells plated on glass-bottomed MatTek dishes were loaded with the ratiometric Ca²⁺ indicator fura-2 AM (10 μM, for 45 min, at 37°C; Molecular Probes, Eugene, OR). Fura-2-loaded cells were imaged on an epifluorescence microscope (Eclipse TE2000-U; Nikon, Tokyo, Japan) equipped with a CCD digital camera (Photometrics CoolSnap HQ2, Tucson, AZ) and a ×20 objective (N.A. 0.45; Nikon, Tokyo, Japan). Changes in fura-2 fluorescence intensities emitted at two excitation wavelengths (340 and 380 nm) were acquired at 1.0 Hz using a Lambda DG-4 filter changer (Sutter Instruments, Burlingame, CA) driven by a computer through Metafluor software (Universal Imaging, West Chester, PA). Values of intracellular Ca²⁺ levels determined from regions of interest placed on cells were obtained from fura-2 ratio images using an in vitro calibration curve. Changes in intracellular Ca²⁺ concentration ≥30 nM were considered as a response.

Statistical analysis. Statistical significance of parameters of interest between groups was in general determined using two-tailed Student’s t-test for unrelated samples. All differences were considered significant at P < 0.05. Unless otherwise stated, all data are expressed as means ± SE.

RESULTS

Vcsa1 expression and plasma levels of sialorphin are decreased in diabetic animals. We (42) have previously demonstrated in the same animal model investigated here that expression of the Vcsa1 gene is significantly reduced in the CSM of 8-wk STZ-diabetic rats compared with AMC. We extended these studies to several other tissues and compared Vcsa1 expression levels in the 8-wk STZ-diabetic rats to AMC (Table 1). As previously reported, the submandibular gland, CSM, and prostate were identified as major sites of Vcsa1 expression (28, 36, 42). In all tissues investigated Vcsa1 expression was significantly reduced after 8 wk of diabetes compared with AMC. We also determined the levels of sialorphin, the mature peptide product of the Vcsa1 precursor protein, in the plasma of 8-wk STZ-diabetic diabetic and AMC rats. Diabetes resulted in a significant decrease (~30% reduction) in the circulating levels of sialorphin (Fig. 1). Both insulin treatment for 1 wk and intracorporal injection of a plasmid expressing Vcsa1 (pVAX-Vcsa1) after 1 wk returned plasma levels of sialorphin to that detected in AMC.

Gene transfer of plasmids expressing sialorphin precursor improves erectile function and lowers BP in the diabetic rat. Erectile function can be determined by measuring the ICP-to-BP (ICP/BP) ratio following cavernous nerve stimulation. As shown in Fig. 2, 8-wk STZ-diabetic compared with AMC animals show decreased erectile function following stimulation of the cavernous nerve. We also observed a decrease in the basal (i.e., no stimulation of the cavernous nerve) ICP/BP ratio in diabetic animals. We have previously reported that in an aging animal model of ED intracorporal injection of plasmids expressing oporphiin homologue genes can improve erectile function (42, 43). Therefore, we investigated if intracorporal injection of plasmids expressing sialorphin gene can also improve erectile function in diabetic animals (Fig. 2). As a positive control for recovery of erectile function, we also intracorporally injected a plasmid expressing the hMaxiK potassium channel. This plasmid (pVAX-hSlo) has been shown to be effective in treating animal models of ED and has been evaluated in Phase I clinical trials for treatment of patients with ED (7, 25, 42). When animals were intracorporally injected with a plasmid expressing Vcsa1 gene (pVAX-Vcsa1), circulating levels of sialorphin returned to the levels seen in AMC (Fig. 3A). This correlated with effects on erectile physiology as shown in Fig. 2. In the absence of cavernous nerve stimulation, we observed a significantly increased basal ICP/BP in both the diabetic and nondiabetic animals treated with pVAX-Vcsa1. At the higher level of stimulation (4 mA) the ICP/BP ratio was increased in pVAX-Vcsa1-treated diabetic animals, such that it was not significantly different compared with non-diabetic AMC (i.e., the pVAX-Vcsa1-treated diabetic animals had normal erectile function). The improvement in erectile function in the diabetic animals was similar to that observed following intracorporal injection of pVAX-hSlo.

STZ-induced diabetes in rats causes a well-documented increase in systemic BP (6, 37, 38). Our experiments confirmed that STZ-diabetic rats experienced an almost 40% increase in BP (Fig. 3B, left). Subsequently we analyzed the effect of intracorporal injection of pVAX-Vcsa1 on systemic BP in the same animals used to determine ICP/BP. Circulating sialorphin was increased to levels comparable to AMC animals 1 wk after
intracorporal injection of plasmids expressing Vcsa1 (Figs. 1 and 3A). In these animals, the increase in circulating sialorphin was associated with a significant decrease (~19%) in the systemic BP (Fig. 3B, right). Another group of diabetic animals was treated with intracorporal injection 100 μg of the sialorphin peptide. The systemic BP was measured before and 1 h following treatment with sialorphin. A significant decrease in BP (~21%) was detected, similarly to the gene transfer of the pVAX-Vcsa1 plasmid (Fig. 3B, middle). A lower dose of intracorporally injected sialorphin (25 μg) did not cause any significant drop in BP or improvement in the ICP/BP response following cavernous nerve stimulation (data not shown).

Sialorphin can enhance C-type-natriuretic induced relaxation of corporal and aortic smooth muscle strips. Human opiorphin and rat sialorphin act as potent endogenous inhibitors of NEP and AP-N (35, 48). Sialorphin, probably through its action as an NEP inhibitor, has been shown to enhance relaxation of CSM tissue by CNP (10). Neither sialorphin nor synthetic NEP inhibitors in the absence of peptide mediators have a direct effect on the tone of isolated smooth muscle tissue (data not shown), providing further support that the role of sialorphin is mediated through its action as an inhibitor of endopeptidase. Synthetic NEP inhibitors have also been demonstrated to relax vascular smooth muscle tissue when used in combination with CNP (21). The reports describing shared mechanisms for the development of ED and vascular smooth muscle pathologies led us to investigate if the effect of sialorphin on the physiology and biochemistry of CSM tissue and erectile function could be transposed to other vascular/smooth muscle systems. As can be seen in Table 2, sialorphin induced a significant increase in the rate of submaximal CNP-induced relaxation in both CSM and aortic muscle strips. In aortic strips, the effect of sialorphin was very pronounced, approximately sixfold greater than in CSM strips.

Sialorphin can modulate changes in intracellular calcium levels induced by bradykinin. Sialorphin has been shown to be a potent NEP inhibitor. Therefore, a possible mechanism by which sialorphin could regulate both aortic and CSM tone may be through potentiation of agonist peptides action on SM cells. Bradykinin is a substrate for NEP, and we hypothesized that...
sialorphin acting as a potent NEP inhibitor may block the degradation of bradykinin and therefore potentiate its ability to induce Ca$^{2+}$ mobilization (30, 34). For these experiments, both rat aortic (A7r5) and CSM cells were loaded with the ratiometric Ca$^{2+}$ indicator fura-2 AM and bradykinin-induced changes in intracellular Ca$^{2+}$ concentration were measured. The optimal working concentration for bradykinin was determined from noncumulative dose-response curves obtained from both CSM and A7r5 cells (Fig. 4, A and E). To better discern a response, a bradykinin concentration smaller than the EC$_{50}$ (the concentration inducing half-maximum response) was selected (3 or 10 nM bradykinin for CSM or A7r5 cells, respectively). We measured the amplitude of Ca$^{2+}$ responses triggered by bradykinin (amplitude of response; Fig. 4, B and F) and the relative number of responding cells (efficacy of response; Fig. 4, C and G) in CSM and A7r5 cells (Fig. 4, B, C, F, and G). Similar effects were observed when CSM or A7r5 cells were preincubated with 1 µM of the synthetic NEP inhibitor thiorphan (Fig. 4, D and H).

### DISCUSSION

Our work adds to the growing body of evidence that common mechanisms are involved in the development of ED and CVD. Diabetes is a risk factor for both CVD and ED, and we have previously reported that in diabetic patients and diabetic animal models the genes encoding opiorphin-related products are downregulated in CSM tissue (1, 42–44, 46). In the present study, we demonstrate that experimental diabetes is also associated with reduced Vcsa1 gene expression in other tissues and with significantly lower plasma levels of sialorphin, the rat opiorphin homologue. We show that overexpression of Vcsa1, the gene encoding sialorphin, leads to significant improvement in both vascular and erectile physiology in diabetic animals, as manifested by reduced BP and increased erectile response. These findings provide evidence that circulating opiorphin-related peptides can affect the biochemistry and physiology of the micro- and macrovascular system. Since opiorphin-related peptides act as NEP and AP-N inhibitors (35, 48), their physiological actions can likely be related to protection of response, which was calculated as the ratio of responding cells/total number of cells imaged in the whole microscope field; Fig. 4, C and G) with or without preincubation with 300 ng/ml of sialorphin for 15 min. Sialorphin did not affect intracellular Ca$^{2+}$ concentration per se (data not shown) but significantly increased both the amplitude and efficacy of bradykinin-induced responses in both CSM and A7r5 cells (Fig. 4, B, C, F, and G). Similar effects were observed when CSM or A7r5 cells were preincubated with 1 µM of the synthetic NEP inhibitor thiorphan (Fig. 4, D and H).

### Table 2. Loss of tension when contracted smooth muscle strips are relaxed with CNP in the presence or absence of sialorphin

<table>
<thead>
<tr>
<th>Tissue</th>
<th>1 µM CNP, Loss of Tension, mean g/min (SD)</th>
<th>1 µM CNP + 1 µg/ml Sialorphin, Loss of Tension, mean g/min (SD)</th>
<th>Fold Increase in Rate of loss of Tension Following 1 µg/ml Sialorphin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSM</td>
<td>0.087 (0.012)</td>
<td>0.13 (0.014)</td>
<td>1.49</td>
</tr>
<tr>
<td>Aorta</td>
<td>0.016 (0.002)</td>
<td>0.151 (0.012)</td>
<td>9.4</td>
</tr>
</tbody>
</table>

Mean (±SD) rate of tension loss was measured from 4 corporal smooth muscle (CSM) strips isolated from 4 different animals. *P < 0.05, significant increase in the rate of tension loss by Students t-test when the strips were treated with 1 µM C-type natriuretic peptide (CNP) and 1 µg/ml sialorphin compared with CNP alone.

![RAT CCSM](image)

**Fig. 4.** Preincubation with sialorphin increases the sensitivity of rat CSM and aorta derived cells to bradykinin. Bradykinin triggers increase in intracellular Ca$^{2+}$ mobilization in CSM cells (Fig. 4A). For these experiments, both rat CSM cells (A) and A7r5 cells (E). These curves were used to determine the optimal concentration of bradykinin to be used in experiments (3 and 10 nM bradykinin for CSM and A7r5 cells). B and F: preincubation for 15 min with 300 ng/ml of sialorphin before addition of bradykinin significantly increased the average amplitude of Ca$^{2+}$ mobilization in CSM cells (B) and A7r5 cells (F). C and G: efficacy of bradykinin-induced Ca$^{2+}$ response (relative number of responding cells) in both CSM cells (C) and A7r5 cells (G) was also significantly enhanced in the presence of sialorphin. D and H: preincubation for 15 min with 1 µM of the synthetic neutral endopeptidase inhibitor thiorphan before addition of bradykinin significantly increased the average amplitude of Ca$^{2+}$ mobilization in CSM cells (D) and A7r5 cells (H). Bars represent means ± SE; n = >200 cells analyzed in each experimental group. *P < 0.05, statistical significance.
peptide signaling molecules that modulate vascular and CSM cells. Indeed, we demonstrated that CNP induced relaxation of both aortic and CSM tissues and that the rate of relaxation was enhanced in the presence of sialorphin. We also show that sialorphin augmented the calcium responses induced by bradykinin in both aortic and CSM cells. The efficacy in improving erectile function and decreasing the BP in diabetic animals could thus be explained by ability of opiorphin-related peptides to modulate smooth muscle tone in the target systems. The synthetic specific NEP inhibitor thiorphan in association with ACE inhibitors has been proposed to treat hypertension and congestive heart failure in diabetic patients. Thiorphan has been shown to enhance the efficacy of the ACE inhibitor in lowering BP and to improve hemodynamic measurements following heart failure by inhibiting both the inactivation of vasodilator and the activation of vasoconstrictor peptides (5, 13) (24). Another NEP inhibitor drug, omapatrilat, developed by Bristol-Myers Squibb also initially showed promise as an antihypertensive agent. However, safety concerns led to it being withdrawn from clinical trials in 2003 (47). Because the opiorphin peptides also act as NEP inhibitors, their mechanism of action could overlap with the synthetic NEP inhibitors and they could be applied to the treatment of similar types of medical conditions. However, the overall physiological effect of opiorphin-related peptides on a particular tissue will ultimately depend on not only on the composition of the peptide receptors but also on the signaling peptides in the environment of the tissue.

Our study suggests the potential use of an opiorphin replacement therapy to treat some of the vascular complications of diabetes, either by direct administration of the peptide or gene transfer of plasmids expressing opiorphin-related genes. Regarding the gene therapy approach, the fact that the rat Vcσ1 gene product can be cleaved according to a common prohormone-related process has to be taken into consideration. Five known biologically active peptide products can be generated, displaying at least three distinct biological activities: analgesia, erectile function, and anti-inflammation (11, 22, 23, 36, 45). Each of these effects may be associated with inhibition of various peptidase activities. Regardless of the putative action of these different peptide products, sialorphin has been demonstrated in previous studies to improve erectile function, and here we show that it can directly lower BP in diabetic animals. Gene transfer of vectors expressing opiorphin may therefore offer the advantage of long term efficacy if used clinically for treating hypertension and ED, as we have previously shown in animal models (27). It has to be mentioned, however, that intracorporal injection of large amounts of plasmids expressing the human SMR3 gene has the potential to result in a priapic-like condition (42, 43). This condition was never observed when opiorphin or sialorphin peptides were administrated systemically or by intracorporal injection, regardless of the dose (Ref. 10 and unpublished observations by K. P. Davies and C. Rougeot). It is conceivable that this priapism-like condition observed in rats following human gene transfer could be induced by the local production of large amounts of unprocessed precursor protein and/or uncharacterized processed peptides that display distinct activity from the mature pentapeptide opiorphin.

Although injecting vectors expressing opiorphin-related genes, or injecting the opiorphin peptide into the corpora might appear to be an unexpected site for administration, the penis represents some advantages compared with other potential sites of injection. It is a readily accessible organ that physiologically expresses large amounts of the gene encoding opiorphin. Although intracorporal injection of plasmid is a painless and an outpatient procedure, it might be expected to engender resistance to its use by the patients. However, the procedure may provide long-term relief of diabetic vasculopathy without the need for daily dosing and significant side effects of present pharmacological treatments. This may tip the balance for patients: a recent survey suggests that intracorporal injection of vectors expressing hMaxiK to treat men with ED would be well accepted by both clinicians and patients, despite the availability oral erectogenic agents if it provides a long-term, safe, and effective treatment (26).

We (4) have previously demonstrated that expression of human opiorphin or opiorphin-related genes is a marker of ED and that their expression increases with ED treatments, such as PDE5 inhibitors, which improve erectile function. These findings lead to the intriguing possibility that decreased expression of opiorphin-related genes in patients with ED may contribute to the development of systemic vascular effects and, conversely, by improving erectile function (for example, through the use of PDE5 inhibitors) will increase plasma levels of opiorphin-related peptides, thereby improving vascular health. It is tempting to speculate that epidemiological studies that suggest ED as a predictor of CVD (5, 39, 41) could also be viewed as providing evidence that ED would indirectly contribute to development of CVD, likely through altered levels of circulating opiorphin-related peptides. This interpretation of our studies would suggest that erectile function is not just an indicator of the overall vascular health, but also makes a key contribution to a man’s health.

ACKNOWLEDGMENTS

G. Calenda and S. O. Suadicani researched data, contributed to the discussion, and reviewed/editied the manuscript; Y. Tong, N. Kanika, and M. T. Tar researched data; A. Melman contributed to the discussion; C. Rougeot contributed to the discussion and reviewed/editied the manuscript; and K. P. Davies contributed to the discussion, wrote the manuscript, and reviewed/editied the manuscript.

GRANTS

Support for the study was provided by National Institute of Diabetes and Digestive and Kidney Diseases Grant R01-DK-077665 (to K. P. Davies).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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