O$_2$-dependent prostanoid synthesis activates functional PGE receptors on corpus cavernosum smooth muscle

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Moreland, Robert B., Hassan Albadawi, Charles Bratton, George Patton, Irwin Goldstein, Abdulmaged Traish, and Michael T. Watkins. O$_2$-dependent prostanoid synthesis activates functional PGE receptors on corpus cavernosum smooth muscle. Am J Physiol Heart Circ Physiol 281: H552–H558, 2001.—We have previously demonstrated that decreased O$_2$ tension inhibits prostaglandin synthesis from human corpus cavernosum smooth muscle cells in static culture over 8–18 h (R. B. Moreland et al., Molecular Urology 2: 41–47, 1998). In this report, an experimental system was designed that allowed determination of the effects of O$_2$ tension changes over the time frame of physiological penile erection. Human corpus cavernosum smooth muscle cells were cultured on microcarrier beads in enclosed stirrer flasks so that rapid changes of O$_2$ tension could be modulated. After 18 h of equilibration at 30–40 mmHg to simulate blood PO$_2$ at penile flaccidity, O$_2$ tension was increased to 100 mmHg for 1 h and then returned to 30–40 mmHg. Media samples were withdrawn for prostanoid synthesis and cell samples were taken for cAMP determinations. After 18 h of 30–40 mmHg PO$_2$ values, prostanoid synthesis by human corpus cavernosum smooth muscle cells was low (0.1–0.7 pmol/10$^6$ cells). When PO$_2$ was increased to 100 mmHg, a rapid increase in PGE$_2$ > PGF$_{2\alpha}$ > PGD$_2$ was observed (thromboxane A$_2$ was undetectable), which peaked at 5.7 pmol PGE$_2$/10$^6$ cells. Increased O$_2$ tension correlated with increased PGE$_2$ and increased intracellular synthesis of cAMP. The prostaglandin G/H synthase inhibitor indomethacin or the E prostanoid (EP2)-selective antagonist AH-6809 each inhibited the O$_2$-tension-dependent increases in cAMP. These data support a role of differential O$_2$ tension in the penis in the smooth muscle synthesis of PGE$_2$, which in turn increases cAMP synthesis via EP$_2$ receptors.

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Penile erection is the end result of corpus cavernosum trabecular smooth muscle relaxation (1, 3, 17). Nitric oxide derived from nerves containing type I nitric oxide synthase (neural NOS) is thought to mediate the initial dilation of the helicine resistance arterioles as well as the trabecular smooth muscle, which results in an increase of arterial blood flow. One of the consequences of this influx of arterial blood is an increase in blood PO$_2$ in the trabeculae from 25–40 mmHg at flaccidity to 90–100 mmHg during erection (9, 25, 26). As the trabecular sinuses relax and fill with blood, intracavernosal pressure and volume increase (1, 9, 17). Venocclusion develops via stretching, compressive forces are initiated by “expandable” trabecular tissue on submucosal venules, and rigid erection ensues. PGE synthesized by the corpus cavernosum smooth muscle cells (14) binds to specific receptors on the smooth muscle and is thought to potentiate smooth muscle relaxation by activating cAMP-dependent pathways (20, 27, 28).

Recent research into the pathophysiology of erectile dysfunction suggests the importance of increased corpus cavernosum connective tissue and a diffuse fibrosis induced in part by arterial insufficiency (11, 19, 26). The key finding in these studies is that corpus cavernosum structure is directly related to erectile function and successful venoocclusion (11, 19, 31). Thus a decrease in the amount of corpus cavernosum trabecular smooth muscle and an increase in connective tissue is correlated with diffuse venous leakage and a failure of the venoocclusive mechanism. The cytokines and vasoactive factors involved in these processes have been investigated in both cell-culture and animal models. Data derived from in vitro cell-culture studies (12, 13) as well as in vivo animal models (16, 18, 29) are consistent with a role for tumor growth factor-$\beta_1$ (TGF-$\beta_1$) as an active agent in diffuse corpus cavernosal fibrosis, which may underlie this pathophysiology. It has further been demonstrated that TGF-$\beta_1$-induced fibrillar collagen synthesis in human corpus cavernosum smooth muscle cells can be suppressed by PGE$_1$ via a cAMP-dependent pathway (12, 13). One hypothesis of erectile dysfunction pathophysiology links the changes in O$_2$ tension to the regulation of vasoactive factors such as PGE that in turn regulate TGF-$\beta_1$-induced connective tissue synthesis via cAMP-dependent mechanisms (11, 12, 19). Although daily periodic oxygenation of the corpora cavernosa occurs by virtue of nocturnal penile tumescence (reviewed in Ref. 11) either in the presence or absence of sexual activity, there is no direct evidence that confirms a link between

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O2 tension, human corpus cavernosum smooth muscle cell PGE synthesis, and cellular signaling (via cAMP) through an autocrine/paracrine loop. In this report, we demonstrate that O2 tension mediates a rapid increase in unstimulated PGE synthesis in the human corpus cavernosum smooth muscle cells, which in turn modulates cAMP synthesis via E prostanoid (EP2) receptors and provides support for an autocrine-loop model.

MATERIALS AND METHODS

Chemicals and supplies. Low-glucose DMEM, antibiotics, nonessential amino acids, and Taq DNA polymerase were obtained from Life Sciences (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Summit Biotechnology (Fort Collins, CO). PGE1 was purchased from Cayman Chemical (Ann Arbor, MI). AH-6809 was purchased from Biomol (Plymouth Meeting, PA). Indomethacin, 3-isobutyl-1-methylxanthine (IBMX), and fatty acid-free BSA were purchased from Sigma Chemical (St. Louis, MO). All other chemicals were of reagent grade and were obtained from commercial suppliers.

Cell culture methods. These studies were approved by the Institutional Review Board at Boston Medical Center, Boston University School of Medicine. Human corpus cavernosum smooth muscle cells were cultured as previously reported (13). Corpus cavernosum biopsies for these cultures were obtained from patients undergoing partial penectomy for penile cancer and from penile prostheses insertion in patients for either Peyronie’s disease (to correct penile curvature), pelvic trauma, or radical retropubic prostatectomy. Patients with penile cancer and Peyronie’s disease were self-reportedly potent before the accident or self-reportedly potent at the time of surgery, whereas the patients for either Peyronie’s disease (to correct penile curvature), pelvic trauma, or radical retropubic prostatectomy were maintained in the medium for the remainder of the experiment. Throughout the experiments, aliquots of media were injected into a blood-gas analyzer to determine the PO2 and PCO2, and the input flow of gases was adjusted accordingly.

ELISA assays for prostaglandins. Samples of media were removed at various times, clarified by centrifugation at 1,000 g for 1 min, frozen in liquid nitrogen, and stored at −70°C until assay. Prostanoids were determined by ELISA (Cayman Chemical; Ann Arbor MI; and R&D Systems; Minneapolis, MN). To prevent decay of PGD2 before centrifugation, low pH ELISA samples were treated immediately with methylxoylm as described by the manufacturer.

RT-PCR detection of EP2 and EP4 receptors. Primers were synthesized to human EP2 (5′ GTTACTGGCTTGATACCGGGG and 3′ CTTCGCGCTCTTCCGGGCAC) (22) and EP4 (5′ GCCA-CACGACCTGTTGGGG and 3′ GCGGTTGGCCAGGACGTGTC) (2) that amplify 438-bp and 421-bp DNA fragments of EP2 and EP4, respectively. The EP2 piece extends from valine-89 in the second transmembrane spanning region to cysteine-234 in the fifth transmembrane spanning region whereas the EP4 piece extends from aspartate-65 in the first extracellular loop to asparagine-202 in the fourth transmembrane spanning region. These primers were chosen such that they flank the exon-intron junctions thereby allowing distinction of genomic DNA and cDNA amplification (21). RT was carried out as previously described (7) using random primers (Life Sciences; Bethesda, MD) and a thermocycler (model 200; MJ Research; Watertown, MA). PCR conditions for EP2 and EP4 were 94°C for 1 min, 65°C for 1 min, and 72°C for 1 min for 35 cycles previous to a 6-min extension step at 72°C. DNA amplification products were separated on 10% nondeaturing acrylamide gels in running buffer containing Tris-HCl, borate, and EDTA (pH 8.30). Confirmation of fragment identity was carried out by digestion of the amplification products with the restriction endonuclease PstI (New England Biolabs; Beverly, MA), which digests the EP2 and EP4 fragments at unique predicted sites.

cAMP synthesis. A 400-μl aliquot of beads was removed at various times using a Gilson Microman positive-displacement pipette through a sealed side port on the experimental flask. The cells were extracted immediately with 0.1 N HCl and were briefly sonicated on ice. The cell homogenate was centrifuged at 11,000 g for 10 min at 4°C. Supernatants were collected into fresh tubes and analyzed for cAMP using a cAMP assay (detergent-compatible protein assay; Bio-Rad; Hercules, CA). cAMP was expressed as picomoles of cAMP per milligram of protein.

RESULTS

Changes in O2 tension modify prostanoid synthesis. Human corpus cavernosum smooth muscle cells grown on microcarrier beads maintained a fusiform morphology when visualized under phase-contrast microscopy, which is consistent with our previous reports of smooth muscle phenotype (13). Cells were cultured for 18 h at...
Po2 = 30–40 mmHg. The rationale for this timing was that 18 h is the longest time at flaccidity one would expect in the absence of sexual activity and in the presence of normal nocturnal penile tumescence (11). Prostanoid synthesis determined in the 30-min interval after this 18-h incubation period showed little prostanoid synthesis of either PGE2, PGF2α, or PGD2 (see Fig. 1). Thromboxane A2 was undetectable. Increasing Po2 to 100 mmHg resulted in a subsequent increase in synthesis of PGE2, PGF2α, and PGD2, which peaked up to 30 min after Po2 was returned to 30–40 mmHg (see Fig. 1). The major prostanoid species produced was PGE2, which was 10- to 20-fold in excess of either PGF2α or PGD2, respectively (see Fig. 1). Indomethacin (10 μM) pretreatment totally abrogated the O2-dependent increase in prostanoid synthesis (data not shown).

**EP receptors in corpus cavernosum smooth muscle.** RT-PCR assays for human EP2 and EP4 mRNA expression revealed positive amplification in human small intestines and kidney (positive controls, lanes 2 and 3; Fig. 2, A and C) with amplification products of 438 bp for EP2 and 421 bp for EP4. A similar result was noted in total RNA prepared from human corpus cavernosum biopsies (4 biopsies out of 4 biopsies total: Fig. 2, lanes 5–8; Fig. 3A) and from total RNA prepared from cultured human corpus cavernosum smooth muscle cells (6 cell cultures positive out of 6 cultures total: Fig. 2, lanes 9–14; Fig. 3, A and C). The identities of these DNA products were established by digestion of the amplification products with the restriction endonuclease PstI, which resulted in the expected 305-bp and 133-bp DNA fragments for EP2 (Fig. 2B) and the 317-bp and 104-bp DNA fragments for EP4 (Fig. 3B). Thus human corpus cavernosum as well as human corpus cavernosum smooth muscle cells in culture express both EP2- and EP4-receptor mRNA.

Increases in PGE2 feed back on functional EP receptors. Having established that there is an O2-dependent increase in nonstimulated PGE2 synthesis and that the Gs-coupled EP2 and EP4 receptors are expressed, we tested whether PGE can activate receptors on human corpus cavernosum smooth muscle cells in an autocrine or paracrine manner. Changes in O2 tension resulted in concomitant increases in intracellular cAMP synthesis (see Fig. 4A) that were coincident with the increase in PGE2 synthesis (see Fig. 4A) that were coincident with the increase in PGE2 synthesis (see Fig. 4A) that were coincident with the increase in PGE2 synthesis (see Fig. 4A). Pretreatment with indomethacin (10 μM), a prostaglandin G/H synthase inhibitor (COX-1 and COX-2), abrogated the O2-tension-dependent increase in cAMP synthesis (see Fig. 4). These results are consistent with the hypothesis that O2-tension-dependent increases in cAMP synthesis are due to prostanoids interacting with Gs-coupled receptors and stimulating smooth muscle cell adenylate cyclase. Because the major increases in unstimulated prostanoid synthesis are in PGE2, it is possible...
that the increase in intracellular cAMP is due to interaction with either EP\textsubscript{2} and/or EP\textsubscript{4} expressed on the corpus cavernosum smooth muscle cells. Pretreatment with the EP\textsubscript{2}-selective antagonist AH-6809 attenuated the O\textsubscript{2}-tension-dependent increase in cAMP synthesis, which under these conditions suggests that most of this response occurs through the EP\textsubscript{2} receptor (see Fig. 4\textsuperscript{A}).

Exogenous PGE\textsubscript{1} (5 \textmu M) had similar effects on cAMP synthesis either in the presence or absence of indomethacin when P\textsubscript{O\textsubscript{2}} = 30–40 mmHg (391.6 ± 7.9 and 383.7 ± 52.8 pmol cAMP/mg protein, respectively), which confirms that changes in O\textsubscript{2} tension alone did not alter signal transduction via the EP receptor system. The differences seen in Fig. 4\textsuperscript{A} are changes in the steady-state concentration of cAMP in the absence of phosphodiesterase inhibitors. When a similar experiment was performed in the presence of IBMX and cAMP levels were measured 5 min after the shift in P\textsubscript{O\textsubscript{2}} from 30 to 100 mmHg, a 300% increase in cAMP levels was observed in the absence but not in the presence of indomethacin (see Fig. 4\textsuperscript{B}).

DISCUSSION

The role of increased O\textsubscript{2} tension during penile erection and the effects of this increase on corpus cavernosum trabecular structure has been proposed and debated (11, 19, 24, 25). The key features of this hypothesis are that the increase in blood O\textsubscript{2} tension during erection results in differential expression of cytokines such as TGF-\beta\textsubscript{1} (induced by O\textsubscript{2} tension consistent with blood P\textsubscript{O\textsubscript{2}} in flaccid cells) and PGE (induced by high O\textsubscript{2} tension), which not only affect smooth muscle tone but also affect connective tissue gene ex-
expression (11–13, 18, 19, 24). It has been demonstrated that TGF-β1 induces 2.5- to 4-fold increases in human corpus cavernosum smooth muscle cell fibrillar collagen synthesis and that this induction of collagen synthesis can be suppressed by PGE1 (13) via a cAMP-dependent pathway (12). Clinically, in two separate prospective studies (19, 31), it has been shown that there is a functional connective tissue/smooth muscle balance in the corpus cavernosum such that below a certain smooth muscle content, diffuse venous leakage and failure of venoocclusion ensues. In one such study (19), we demonstrated functional TGF-β1 responses in cultured smooth muscle cells regardless of the percentage of smooth muscle in the original biopsy. These data suggest that either the loss of synthesis of modulators that inhibit the actions of TGF-β1 are not present in the tissue, or the factors responsible for the pathology are lost when the smooth muscle cells are cultured in vitro. We went on to suggest that nocturnal penile tumescence during rapid eye movement sleep could provide the daily periodic oxygenation necessary to maintain a functional balance (11, 19). This hypothesis integrates individual results from tissue culture and animal models as well as clinical data to formulate the concept of a paracrine-autocrine loop (11): O2 tension regulates the synthesis of autacoids, which in turn regulate cAMP and hence connective tissue expression. However, the principle of an autocrine loop regulated by O2 tension has yet to be demonstrated in human corpus cavernosum smooth muscle cells in any experimental system.

In this report, we have shown that PGE2 is the major prostanoid synthesized and released by human corpus cavernosum smooth muscle cells in response to increases in O2 tension. Furthermore, simply increasing PO2 from the conditions observed during flaccidity (30–45 mmHg) to those observed during erection (100 mmHg) results in an eightfold increase in unstimulated prostanoid synthesis. These observations confirm the qualitative results observed in static smooth muscle cell cultures (14) and extend those observations to time frames consistent with those reported for physiological penile erection (9, 10). In this study, synthesis and release of PGD2, PGE2, and PGF2α were observed with no detectable release of thromboxane A2. Although the levels of synthesis were low compared with PGE2, this is the first demonstration of PG2 synthesis in human corpus cavernosum. Furthermore, the prostanoid profile in this study differs from the reports of rabbit corpus cavernosum in organ culture where the major prostanoids produced were PGE2, PGF2α, and PGF2β with detectable amounts of thromboxane A2 (4). These differences may reflect changes in methodology for assaying PGD2 (not determined in the organ-bath study) as well as the presence of corpus cavernosum endothelium in the rabbit organ-culture experiments. Both PGL2 and thromboxane A2 may be synthesized by the endothelium. The results in this study have special significance because the major prostanoid produced by smooth muscle is PGE2, which has the potential to act as a local mediator in erection, further facilitating corpus cavernosum smooth muscle relaxation.

Prostanoids exert their effects on cells and tissues by binding to specific G protein-coupled receptors (15). In this study, we show that the O2-dependent increases in smooth muscle cAMP synthesis are due to prostanoids by virtue of the inhibition of this effect by indomethacin pretreatment. Of the relaxatory prostanoids, only PGE relaxes human corpus cavernosum (1, 8), probably via cAMP-dependent pathways (12, 20, 27, 28). It is known that PGE binds to specific membrane receptors, of which there are four pharmacological subclasses (15, 21). Of the seven cloned human PGE (EP) receptors, only the EP2 and EP4 subtypes increase cAMP levels upon PGE binding in human cells and tissues (2, 15, 21, 22). Therefore, because PGE1 elevates cAMP in human corpus cavernosum smooth muscle cells (12, 20, 27, 28), either functional EP2 and/or EP4 must be present on these cells. In this report, we have demonstrated the expression of both EP2 and EP4 mRNA in human corpus cavernosum biopsies as well as cells (see Figs. 2 and 3). Although EP4 may play an as-yet undefined role, pretreatment with the EP2-selective antagonist AH-6809 (32) blocks O2-tension-induced increases in smooth muscle cAMP synthesis (see Fig. 4A). These results demonstrate that the O2-dependent prostanoid-related increases in smooth muscle cAMP synthesis occur most likely by PGE2 exerting an effect via EP2 receptors.

What evidence exists for an autocrine-paracrine loop? A working hypothesis is shown in Fig. 5. In such a hypothesis, at conditions where the penis is flaccid and PO2 = 25–40 mmHg, the synthesis of cytokines such as TGF-β1 are favored. In reports in static cultures of smooth muscle cells, increased TGF-β1 mRNA expression was observed as early as 8 h but was not pronounced until 18–24 h (14). These results suggest a low level of synthesis of this cytokine, which may become pronounced under conditions of prolonged ischemia or in the absence of nocturnal penile tumescence-mediated events. Nevertheless, TGF-β1 immunoreactivity is detectable in human corpus cavernosum biopsies, even in specimens with a normal percentage of trabecular smooth muscle (14). Upon secretion as a complex of TGF-β1 and latent peptide, inactive TGF-β1 must be activated by an as-yet unknown mechanism. Activated TGF-β1 can bind to the two high-affinity, signal-transducing receptors on corpus cavernosum smooth muscle cells or to the low-affinity nonsignaling type III receptor. Binding to high-affinity TGF-β receptors induces connective tissue synthesis as well as induces TGF-β1 mRNA expression and either increased expression or availability of TGF-β receptors. In other cell types (5, 6), TGF-β1 also induces prostaglandin G/H synthase (COX-1 and COX-2) mRNA and protein production as well as PGE2 synthesis (5), which in this model may represent a negative-feedback loop.

In static cultures of human corpus cavernosum smooth muscle cells, TGF-β1 induces a fivefold increase in COX-1 mRNA 24 h after treatment (R. B. Moreland and Y. H. Huang, unpublished observations). The sig-
nificance of a negative-feedback loop that may affect TGF-β1 activity in these cells remains to be investigated.

During erection, blood PO₂ increases to 90–100 mmHg (9). Under these conditions in our experimental model, PGE₂ was the major prostanoid produced and it binded EP2 receptors on the smooth muscle cells and elevated cAMP. This increased cAMP synthesis may have a dual purpose. First, it may act as a secondary mediator of erection, locally enhancing smooth muscle relaxation (1, 8, 27). Second, this increased cAMP may have transcriptional and posttranscriptional effects of inhibiting connective tissue synthesis as well as TGF-β1 mRNA expression. It is interesting to note that the basal levels of cAMP under in vitro flaccidity conditions is 20 pmol/mg protein, whereas in the presence of indomethacin, it is one-half that amount. Although it has been established that cAMP will inhibit TGF-β1-induced collagen synthesis in human corpus cavernosum smooth muscle cells, it remains to be determined what level of intracellular cAMP is necessary to maintain a balance between the processes of connective tissue synthesis and accumulation and a functional trabecular smooth muscle/connective tissue ratio. It should be noted that exogenous PGE1 added after 60 min of PO₂ of 30 mmHg gave the same increased synthesis of cAMP either with or without indomethacin. This implies that if PGE is available, the receptors are functional regardless of O₂ tension or inhibition of prostaglandin G/H synthase. In examining the effects of O₂ tension in static cultures of human corpus cavernosum smooth muscle cells on TGF-β1-induced collagen synthesis, the signaling pathways by which TGF-β1 induced and PGE1 suppressed this synthesis remained functional regardless of O₂ tension (14). The overall differences in collagen synthesis at high and low O₂ tension in that study could be attributed to O₂-dependent collagen posttranslational modification (e.g., prolyl and lysyl hydroxylation) (14). In contrast, AH-6809 suppressed cAMP synthesis in the first 30 min after PGE1 stimulation, as would be expected of an EP2-competitive antagonist if this receptor mediates the response. By 60 min, this inhibition was not detectable. Taken together, these results are consistent with the hypotheses that in human corpus cavernosum smooth muscle cells the O₂-tension-mediated increases in cAMP are mediated by PGs (most likely PGE) and that the EP2 receptor is the predominant subtype mediating this effect (see Fig. 5). Finally, nocturnal penile tumescence occurs in normal healthy males three to six times each night coincident with rapid eye movement sleep (10, 11). A recent study found that these episodes could last from 10 to 50 min in duration (average 17 min) in normal males (10). In this study, we demonstrate a prostaglandin G/H synthase-dependent increase in cAMP levels in as little as 5 min after changes in PO₂ from 30 to 100 mmHg (see Fig. 4B). Although it is difficult to extrapolate the in vitro cell-culture data in this report to the in vivo...
physiology, it is possible that such mechanisms could play a role to maintain potency.

In conclusion, we have developed a model to study rapid O2-tension changes in human corpus cavernosum smooth muscle cells. We show that there is an O2 tension-dependent increase in unstimulated prostaglandin synthesis, a concomitant prostaglandin-dependent increase in intracellular cAMP synthesis, and that this cAMP synthesis is mediated through the EP2 receptor. These data suggest that PGE2 may play a role in the physiology of erection and, taken together with previous studies, may be important in the maintenance of a functional smooth muscle-connective tissue ratio.

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