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Metabolic Studies on the Rabbit Corpus Cavernosum

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ABSTRACT: Erectile function (erection and detumescence) involves the complex interaction of direct neuronal stimulation of corporal smooth muscle, neurohumoral release of specific endothelial contractile and relaxant factors, and secondary modulation by a variety of putative neuropeptides and vasoactive modulators. The net result is a rapid and sustained relaxation of the smooth muscle elements during erection and contraction of the smooth muscle during detumescence. Proper function of the corporal tissue is dependent upon cellular metabolism of glucose and the generation of cellular energy in the form of high energy phosphates. The current study characterizes the following metabolic parameters of the rabbit corpus cavernosum: Tissue concentrations of creatine phosphate (CP), ATP, ADP, and AMP; maximal rate of glucose metabolism to lactic acid and CO_2; and activities of the enzymes creatine kinase (CK), citrate synthase, and malate dehydrogenase. For comparative purposes only, bladder smooth muscle preparations were analyzed simultaneously with and under the same conditions as the corpus cavernosum.

The results are as follows: The concentrations of ATP and CP in the corpora were significantly lower than the concentrations in bladder. In the corpora, the tissue concentration of CP was lower than the tissue concentration of ATP, whereas the concentration of CP in the bladder was higher than the concentration of ATP. The rate of glucose metabolism to lactic acid and to carbon dioxide was similar for both bladder smooth muscle and corpus cavernosum. The maximal enzymatic activity of the mitochondrial enzyme citrate synthase was similar for both tissues; similarly, there was no significant difference in the activity of malate dehydrogenase between the two tissues. However, the CK activity of the bladder smooth muscle was significantly greater than the activity in the corpus cavernosum. One important factor that must be considered is that only 34% of the corpus cavernosum is smooth muscle, the balance being extracellular matrix, endothelium, and cytoskeletal elements. Thus, the differences between these two tissues may well be related to the different structural components.

Key words: Erection, corpus cavernosum, autonomic agents, energetics, adenine nucleotides.

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Smooth muscle function is not only dependent on the integrity of its autonomic innervation but is also intimately related to intracellular metabolism. Although there are virtually no direct studies on corporal smooth muscle metabolism, detailed studies on vascular, urinary bladder, gastrointestinal, and other smooth muscle systems can be used to make generalizations concerning the importance of intracellular metabolism and energetics of supporting corporal smooth muscle function (Butler et al, 1977; Siegman et al, 1980; Stephens and Wrogemann, 1983; Coburn and Fillers, 1989; Paul et al, 1989; Hai et al, 1991; Hai and Murphy, 1992).

The metabolic properties of vascular smooth muscle have been studied extensively. (Paul et al, 1973; Hellstrand et al, 1977; Homsher et al, 1978; Chang and Detar, 1980; Detar 1980; Wendt, 1989; Levin et al, 1991b). All smooth and skeletal muscles can obtain energy from both aerobic and anaerobic metabolism. One notable difference between smooth muscle and skeletal muscle systems concerns the ratio between aerobic and anaerobic glycolysis during resting conditions. In smooth muscle, aerobic glycolysis accounts for approximately 50–70% of glucose metabolism, whereas in skeletal muscle at rest virtually 100% of glucose is metabolized to CO_2 (Hamsher and Kean, 1978; Stephens and Wrogemann, 1983; Paul et al, 1989; Hai et al, 1991). In addition, current studies have demonstrated that the energy source for specific cellular functions in vascular smooth muscle (contraction, ion movements, phosphorylation reactions, etc.) depends on the cellular localization (compartmentation) of specific enzyme systems mediating glycolysis, glycogenolysis, oxidative phosphorylation, and specific ATPases. At the present time, no data concerning the basal metabolism or enzyme compartmentation of corporal tissue are available.

The contractile response of the urinary bladder to pharmacological stimulation (benthanechol, methoxamine, KCl) utilizes metabolic energy, whereas bladder smooth muscle relaxation reduces the energy utilization of the tissue (Levin et al, 1987, 1988, 1991a). Current investigations from our laboratory indicate that both re-

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ceptor-mediated contraction and relaxation of the corpus cavernosum increase the utilization of metabolic energy (Hypolite et al, 1993). The energetics of these processes in the corpora are not well understood.

The smooth muscle of most organs is innervated by arterial blood. The corpus cavernosum, however, is one of the only smooth muscle organs that functions physiologically at various PO_2 levels (Lue and Tanagho, 1987; Broderick et al, 1992; Kim et al, 1992). Under resting conditions, basal tone (alpha-adrenergic) is relatively high, maintaining the penis in a flaccid state (Lue et al, 1983; Persson et al, 1989). During the initiation of erection, arterial blood flow into the corpus cavernosal tissue is increased, which increases the level of oxygenation to approximately that of arterial blood. During sustained erection, blood flow through the corpora is reduced, and the PO_2 is progressively reduced to that of venous blood (Lue et al, 1983, 1987; Persson et al, 1989; Broderick et al, 1992; Kim et al, 1992). During prolonged erection (low-flow priapism) the state of oxygenation can be reduced to near anoxic levels (Broderick and Lue, 1988; Lue and McAninch, 1988).

In view of the unique contractile/relaxation properties of the corpora (Azadzoi et al, 1988, Saenz de Tejada et al, 1988; Broderick et al, 1991) and the wide ranges in tissue oxygenation, it is reasonable to assume that the cellular metabolism of the corpora might be quite different from that of other smooth muscle systems.

The current study was designed to obtain basic data regarding the levels of adenine nucleotides present in the rabbit corpus cavernosum, the rate of glucose metabolism, and the enzymatic activities of creatine kinase (CK), citrate synthase, and malate dehydrogenase (three important enzymes in cellular energetics and metabolism). Citrate synthase is found exclusively in mitochondria, malate dehydrogenase has both mitochondrial and cytosolic functions, and CK is primarily a cytoplasmic enzyme. For comparative purposes only, bladder smooth muscle preparations were analyzed simultaneously and under the same conditions as corporal tissue. This should not be taken to mean that corporal tissue and bladder smooth muscle are related in any way.

Materials and Methods

Tissue Source

For each male White New Zealand rabbit the bladder was removed and placed in Tyrode's buffer at 37°C and equilibrated with 95% O_2 and 5% CO_2 for 1 hour. At this time the smooth muscle was dissected free of the mucosa, lamina propria, and serosa. In the same rabbit, the penis was removed at the level of the attachment of the corporal bodies to the ischium. The grossly dissected organ preparation was then placed in oxygenated Tyrode's solution. At this time, most of the overlying skeletal muscle was removed with care not to damage the underlying tunica albuginea. Once fully exposed, a slit was made in the proximal end of the tunica and extended distally. The corpus cavernosum was sharply dissected free of the tunica bilaterally.

High-Performance Liquid Chromatography (HPLC) Analysis of Adenine Nucleotides and Creatine Phosphate (CP) (Levin et al, 1989)

Six individual corpus cavernosa (approximately 50 mg each) and similar size strips of isolated bladder body smooth muscle were further equilibrated in buffer for 1 hour and then rapidly frozen in liquid N_2 until analyzed for nucleotides. On the day of the analysis, each tissue was ground to a fine powder in 200 μl of 0.5 M perchloric acid in a mortar precooled with liquid N_2. Beta–gamma-methylene ATP was added to the frozen powder as an internal standard. After thawing and centrifugation, each supernatant was neutralized with 5 M K_2CO_3. ATP, ADP, AMP, and (CP) were assayed by HPLC using a Beckman model 125 pump and model 166 UV detector. Separation was achieved using a Partisil SAX anion-exchange cartridge (Waters, 8 mm × 100 mm) with a guard column packed with Whatman pellicular anion-exchange medium and a precolumn packed with silica. CP was measured isocratically at 214 nm using an ammonium phosphate buffer (56 mM, pH 4.8). ATP, ADP, AMP, and beta–gamma-methylene ATP were measured isocratically at 254 nm using an ammonium formate buffer (0.5 M, pH 3.7).

CO_2 Measurement (Lin et al, 1989; Kato et al, 1990)

Six individual corpora and six isolated strips of bladder smooth muscle were placed in scintillation vials containing a final volume of 2.0 cc Typrode's–Hepes buffer with the following composition: NaCl, 0.126 M; KCl, 0.0027 M; MgCl, 0.0005 M; NaH_2PO_4, 0.0003 M; Hepes, 0.020 M. To this solution was added a fixed concentration of ^{14}C-glucose (1.0 mg/ml) and $CaCl_2$ (0.265 mg/ml). The vials were gassed for 30 seconds with 100% O_2 and fitted with rubber stoppers equipped with plastic wells containing 0.2 ml phenylethylamine for absorption of CO_2 produced by the tissue. The vials were incubated for 1 hour at 37°C. At the end of 1 hour, 0.5 ml of 1.25 M perchloric acid was added to the vials. The flasks were incubated for a further 30 minutes to allow for complete absorption of CO_2. The wells containing the CO_2 were then added to scintillation vials containing 10 ml Cytoscint (ICN Biomedicals, Inc.) for measurement of radioactivity in a Packard scintillation counter. Based on the specific activity of the glucose substrate, the counts were converted to μmol/mg wet weight of tissue. In all experiments, control vials were incubated without tissue under the same conditions as vials containing tissue.

Lactic Acid Measurement

The concentration of lactic acid within the incubation medium was measured spectrophotometrically. The following is the assay procedure: 1.0 ml lactic acid buffer (200 mM glycyl-glycine, 300 mM hydrazine sulfate, 1.0 mM EDTA, pH 9.1), 100 μl NAD (0.15 mg/ml), 200 μl incubation medium or standard, 10 μl lactate dehydrogenase (diluted 1:2). After mixing, the solution was incubated for 1 hour at 37°C. At the end of the incubation

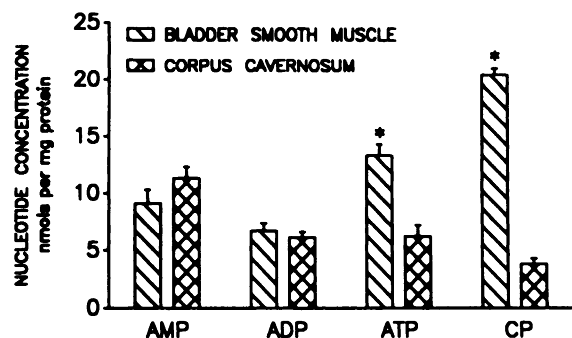

FIG. 1. Adenine nucleotide concentrations of the rabbit corpus cavernosum in comparison to the levels in bladder smooth muscle. Each bar is the mean ± SEM of four to six individual preparations. *Significantly different from the corpus cavernosum, $P < 0.05$.

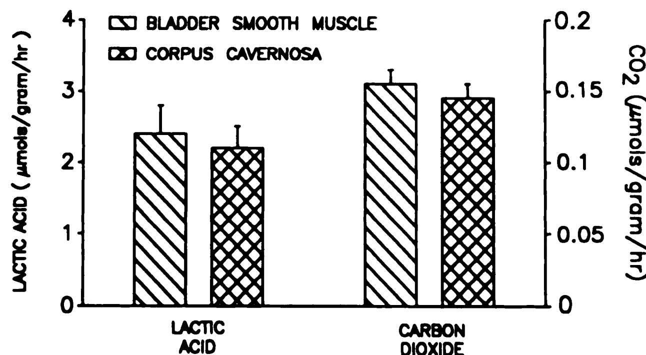

FIG. 2. Metabolism of glucose to lactic acid and CO_2 in rabbit bladder smooth muscle and corpus cavernosum. Each bar is the mean ± SEM of four to six individual preparations.

period, aliquots of the assay mixture were transferred into a cuvette and read at 340 nm in a Hitachi 100-40 spectrophotometer. The lactate concentration is directly proportional to the increase in NADH, which is read as an increase in optical density at 340 nm.

Measurement of Citrate Synthase and Malate Dehydrogenase Activity (Haugaard et al, 1992)

Six isolated corpora and six isolated strips of bladder smooth muscle were homogenized individually in 10 volumes of 0.225 M mannitol–0.075 M sucrose using a Polytron homogenizer. The homogenates were filtered through gauze, and the liquid fraction was kept on ice for analysis of enzymatic activity. Malate dehydrogenase was determined as described previously (Lin et al, 1989). Aliquots of homogenates were incubated in the presence of oxaloacetate and NADH. The rate of oxidation of NADH was measured spectrophotometrically by recording the absorbance at 340 nm every 0.5 minutes. The complete system is as follows: 2.0 ml 0.1 M triethanolamine buffer (pH 7.6), 50 µl 10 mM oxaloacetate, 40 µl NADH (4.0 mg/ml + 40 mg/ml $NaHCO_3$), 100 µl 0.1 M $MgCl_2$, 50- or 100-µl aliquots of homogenates. The temperature of the reaction mixture was 25°C.

Citrate synthase was determined as described previously (Haugaard et al, 1992). Aliquots of homogenate were added to a cuvette containing 1.0 ml 0.05 m Tris buffer (pH 7.6), 100 µl 1.0 mM 5,5'-dithio-bis-(2 nitrobenzoic acid) (DTNB) or Ellmans reagent, 50 µl 10 mM oxaloacetate, and 30 µl 12.3 mM acetyl-coenzyme A. The free coenzyme A generated reacts with DTNB to form a yellow compound, which is measured spectrophotometrically at 412 nm. The change in optical density is read at 30-second intervals over a period of 5.0 minutes at 25° C.

Measurement of CK Activity (Levin et al, 1991a)

Six individual corpora and six isolated strips of bladder body smooth muscle were cut up with a small scissors and homogenized with a Polytron homogenizer (Brinkman Instruments) in approximately 10 ml of buffer. Each homogenate was filtered through two layers of 4" & 4" nylon mesh into a centrifuge tube. The homogenate was centrifuged at 10,000 × *g* for 30 minutes at 4° C; the supernatant was used for CK activity determinations (1 ml of supernatant was frozen for protein determination).

The soluble fraction (supernatant) was allowed to sit 15 minutes at room temperature to allow any endogenous ATP to degrade before the assay was begun. The ATP level was checked using the method of bioluminescence measurement described below; then the sample was put back on ice.

Purified luciferase–luciferin was dissolved in MOPS-Tris (10 mM MOPS, 10 mM Tris, pH 7.6, containing 0.05 mg/ml dithiothreitol and 1 mg/ml BSA) to a concentration of 0.5 mg/ml. The luciferase–luciferin was allowed to sit at room temperature for 60 minutes to reduce the background luminescence.

ATP was quantitated using a Berthold Lumat Luminescence Biometer: 100 µl of sample to be assayed was inserted into the biometer; 100 µl of luciferase–luciferin reagent was automatically injected into the sample tube through the injection port. The concentration of ATP in the tube was calculated from a standard ATP curve. All samples and standards were performed in duplicate.

CK activity was determined as follows: 100 µl of each soluble fraction (various dilutions) was added to 100 µl assay buffer (25 mM glycine buffer, pH 6.8, containing 10 mM $MgCl_2$, 50 µM ADP, and varying concentrations of CP [3.9–1,000 µM]) in a test tube, vortexed, and the mix incubated for 1 or 2 minutes. Incubation was stopped by adding 10 µl of the CK incubation mixture to 90 µl H_2O in a biometer tube, vortexing, and immediately measuring the ATP concentration.

Preliminary studies on the rate of ATP generation as a function of time demonstrated that the reaction was linear up to a minimum of 5 minutes, after which the activity decreased slowly. The lower concentrations remained linear for a proportionally longer time period.

Results

Figure 1 shows the tissue concentrations of ATP, ADP, AMP, and CP in the corpora and the bladder smooth muscle. In the bladder, the tissue concentration of CP is significantly greater than ATP, and the concentrations of both CP and ATP are significantly greater than the concentrations of ADP and AMP. In the corpora, the tissue concentrations of CP and ATP are not significantly dif-

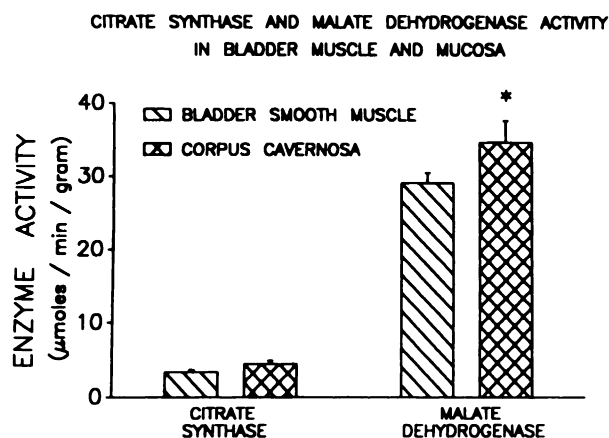

FIG. 3. Enzymatic activities of malate dehydrogenase and citrate synthase of rabbit bladder smooth muscle and corpus cavernosum. Each bar is the mean ± SEM of six individual preparations.

ferent from each other or from the concentrations of AMP and ADP. Comparatively, tissue concentrations of both CP and ATP in the bladder are both significantly and substantially greater than the respective concentrations of CP and ATP in the corpora.

The basal rate of glucose metabolism to CO_2 and lactic acid was similar for both bladder smooth muscle and corpus cavernosum (Fig. 2). The maximal enzymatic activity of citrate synthase was similar for both tissues, whereas the activity of malate dehydrogenase was slightly greater in the corpora (Fig. 3). Figure 4A compares the CK activities of corpus cavernosal tissue and bladder smooth muscle. The kinetic analysis of corpora tissue and bladder smooth muscle is presented in Figure 4A,B. Both tissues presented clear evidence of biphasic enzyme activity. Both tissues show both high affinity and low affinity components of CK activity. However, the maximal enzyme activities of the bladder smooth muscle (both high and low affinity forms) were significantly greater than the activities of the CK isolated from the corpus cavernosum.

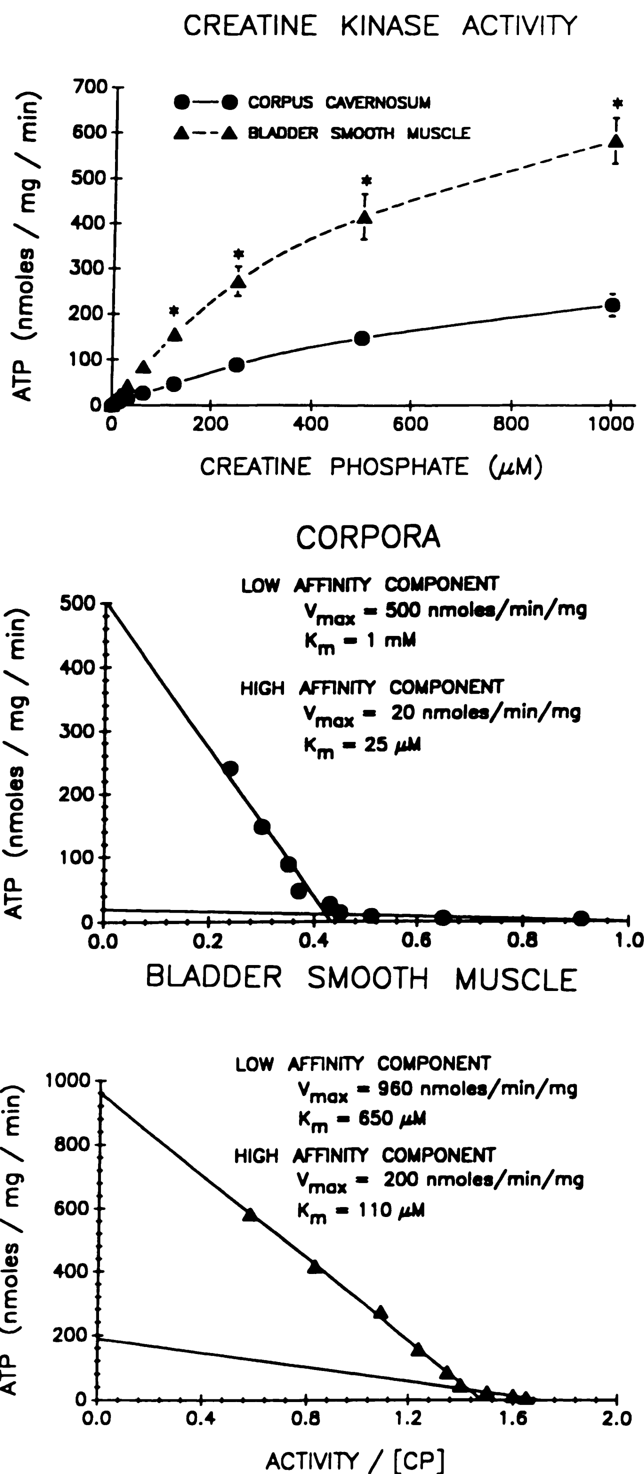

FIG. 4. (A) Enzymatic activity of creatine kinase in bladder smooth muscle and corpus cavernosum. Each bar is the mean ± SEM of six individual preparations. **(B)** The Hofstee plots of creatine kinase activity. Lines are drawn by computer analysis utilizing the ChemFit program. The points are best described by a two affinity site system.

Discussion

Smooth muscle function is dependent on cellular metabolism and the production of high energy phosphates. Although smooth muscle contraction (interaction of contractile filaments) utilizes cytosolic preformed ATP, other specific cellular processes can utilize energy derived from mitochondrial respiration, and energy derived from glycolysis (anaerobic metabolism) (Paul et al, 1973, 1989; Butler et al, 1977; Homsher and Kean, 1978; Stephens and Wrogemann, 1983).

The rate of CO_2 generation and lactic acid formation for the corpus cavernosum was similar to that of bladder smooth muscle. In addition, the ratio of lactic acid to

carbon dioxide for both tissues was similar. This would suggest that the enzymatic pathways involved in the metabolism of glucose were similar for both tissue types. Consistent with this finding, the maximal activities of citrate synthase (a mitochondrial enzyme) and malate dehydrogenase (cytosolic and mitochondrial) were similar for both tissue types.

It is interesting that even though glucose metabolism is similar for both tissues, the cellular concentration of high energy phosphates is substantially lower in the corporal tissue. The bladder has substantially higher cellular concentrations of ATP and CP, whereas the concentrations of ADP and AMP were similar for both tissues. It should be noted that these values were obtained in tissues incubated for 1 hour in oxygenated buffer containing glucose. Previous studies in bladder demonstrated that the intracellular concentrations of ATP and CP in bladder tissue decreased by approximately 20% in incubated tissue (with the simultaneous increase in the concentrations of AMP and ADP) when compared to fresh frozen tissue. In these studies, the concentration of high energy phosphates decreased within 15 minutes of the start of incubation and then remained stable for several hours (Levin et al, 1991a; Haugaard et al, 1992).

The differences in high energy phosphate levels between bladder and corpora may be related to the relative concentration of smooth muscle in the tissue preparation. The bladder preparation was dissected free of mucosa, and thus the major component in the tissue preparation was smooth muscle. The corpora is a composite of a variety of cell types including smooth muscle, endothelial cells, and connective tissue. Recent morphometric studies indicate that only 34% of the corporal mass is smooth muscle, 65% is extracellular matrix, and 1.4% is endothelial cells (Levin et al, 1982; Lue et al, 1988). Unfortunately, there is no way to dissect the smooth muscle away from the matrix in the corpora. In general, the corpus cavernosum should not be considered a "smooth muscle" in the same context as the bladder or vascular smooth muscle. Thus, the data presented in this study for the corpora are the characteristics of the corpora as a tissue.

We are presently involved with studies comparing the metabolism of smooth muscle tissue isolated from bladder with smooth muscle tissue cultures derived from the dissected smooth muscle tissue. These studies demonstrate that the enzyme activities of the smooth muscle cultures are not the same as the tissue from which they were derived. Thus, isolation of corpus cavernosal smooth muscle via tissue culture techniques would not be of great value in these metabolic studies.

Because the concentration of high energy phosphates is significantly lower in non-smooth muscle tissues elements than in smooth muscle, it was not unexpected that the tissue concentration of high energy phosphates was significantly lower in corporal tissue than in bladder smooth muscle. The relatively high CK activity of the bladder smooth muscle (as compared to the corpora) is consistent with the significantly higher levels of ATP found in the bladder smooth muscle. CK exists primarily as a cytoplasmic enzyme; however, there is a mitochondrial CK with different properties. The high affinity enzyme component of both corpora and bladder may represent the mitochondrial component. Future studies will be directed at studies on mitochondrial CK.

One interesting point is that even though the basal concentrations of ATP and CP were lower in corporal tissue, the rate of CO_2 production, lactic acid formation, and malate dehydrogenase–citrate synthase activities were similar for the two tissues. This may indicate that the relative turnover of high energy phosphates in the smooth muscle components of the corpora may be relatively high.

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