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TESTOSTERONE AND HYDROCORTISONE-STIMULATED RESPONSES OF REDUCED PYRIDINE NUCLEOTIDE FLUORESCENCE IN PROSTATES CULTURED FROM CASTRATE RATS

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SUMMARY

1. Addition of testosterone to prostate organ cultures containing 0.1 % glucose is followed after 40 to 60 min by a gradual increase in reduced pyridine nucleotide fluorescence. In several experiments oscillations were superimposed upon the increasing steady-state fluorescence level. The average increase in fluorescence was 8.5 %.

2. When a similar experiment was done in medium containing 0.05 % glucose only a 2 % increase in fluorescence was seen.

3. A single addition of glucose to the testosterone-pretreated prostate resulted in an increase in reduced pyridine nucleotide fluorescence of 6 %. Stepwise additions of both equal and larger amounts of glucose produced similar but smaller change in fluorescence.

4. Hydrocortisone addition to prostates produced a cycle of fluorescence which began within 2 min, attained a maximum within 5–12 min and was complete within 20–30 min. Addition of testosterone after the hydrocortisone response is complete stimulates an immediate increase in fluorescence to a level 10 to 15 % higher than that seen before testosterone addition.

5. N_2–air cycles before hydrocortisone and testosterone showed a change in fluorescence of 4 %. After hydrocortisone and testosterone the change in fluorescence in response to N_2 was 9 %. This indicated that a doubling of the amount of pyridine nucleotide linked to respiration occurred. However, the increase in the steady-state fluorescence was 15 %, indicating that there was an equivalent increase in glycolytic pyridine nucleotide.

6. The addition of 17β-estradiol produced an immediate small increase in the fluorescence signal which can be explained by its intrinsic fluorescence.

INTRODUCTION

The work of LASNITZKI, DINGLE AND ADAMS has shown that prostates from adult rats may be macerated and cultured on a stainless-steel grid[1]. These organ

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cultures are responsive to the growth-promoting effects of testosterone and growth-inhibiting effects of 17β-estradiol.

We have previously shown that metabolic transients in the prostate were seen following the injection of testosterone into castrate rats[2]. These transients could be monitored directly by means of a tissue fluorimeter or by means of metabolite assays. These experiments were subject to the usual criticisms that one encounters when working *in vivo*; that the results may be products of the effects of stress and the presence of other hormones, some of them with androgenic properties, whose levels are not under experimental control.

In this paper we present the results of experiments on prostates of castrate rats cultured according to the general procedure of LASNITZKI, DINGLE AND ADAMS[1]. We observed changes in reduced pyridine nucleotide fluorescence using an improved compensated fluorimeter[3]. Our observations lasted 60 to 90 min after the addition of testosterone to the culture. The effects of hydrocortisone and of 17β-estradiol were followed for about 30 min. Control experiments were also done by adding to the culture either the vehicle in which the steroid hormones were suspended or by making no addition. Such control experiments test the electronic stability of the apparatus, and indicate any fluorescence quenching due to tissue damage produced by the 366 mμ mercury arc used to stimulate fluorescence.

MATERIALS AND METHODS

Prostates of rats which had been castrated 20 to 30 days previously were removed aseptically, minced with fine scissors in a drop of Eagles medium and placed on a Frankle No. W-4829 stainless-steel grid (obtained from the Joseph E. Frankle Co., Philadelphia, Pa., U.S.A.). The level of the medium in a 5-cm petri dish containing the grid was kept just touching, the under surface of the tissue. All media contained 0.035 mg/ml penicillin and streptomycin and 20 % calf serum. The upper surface of the tissue was exposed to the water-saturated gaseous environment (5 % CO_2, 95 % air), of an incubator maintained at 37°. The cultures used for experimental studies were spread out on the grid surface. After 3 days of incubation, growth of tissue between the cut edges of the culture was noted as was a yellowing of phenol red in the culture medium. The prostates were cultured from 1 to 3 days. Prior to each experiment the medium containing phenol red was replaced with medium free of the dye. In dye-free medium the fluorescence artifact due to testosterone or hydrocortisone addition was small, 3 to 5 %, and returned quickly to the level seen prior to such addition. Testosterone was Squibb Testryl made up in 50 % ethanol and hydrocortisone and 17β-estradiol were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. and dissolved or suspended in 50 % ethanol.

Each petri dish containing a culture was mounted in a basket made of coiled 1/4-inch copper tubing which had been soldered together. Liquid (96 % water–4 % ethylene glycol) maintained at 37° by a constant temperature bath was pumped through this basket of coiled copper tubing at 26 l/min. The total volume of the copper tubing was swept out by 37° fluid about 200 times per min. The experimental apparatus is shown in Fig. 1.

The viability of the cultures was tested by visual monitoring of acid production in phenol red-containing medium, by the responsiveness of reduced pyridine nucleotide

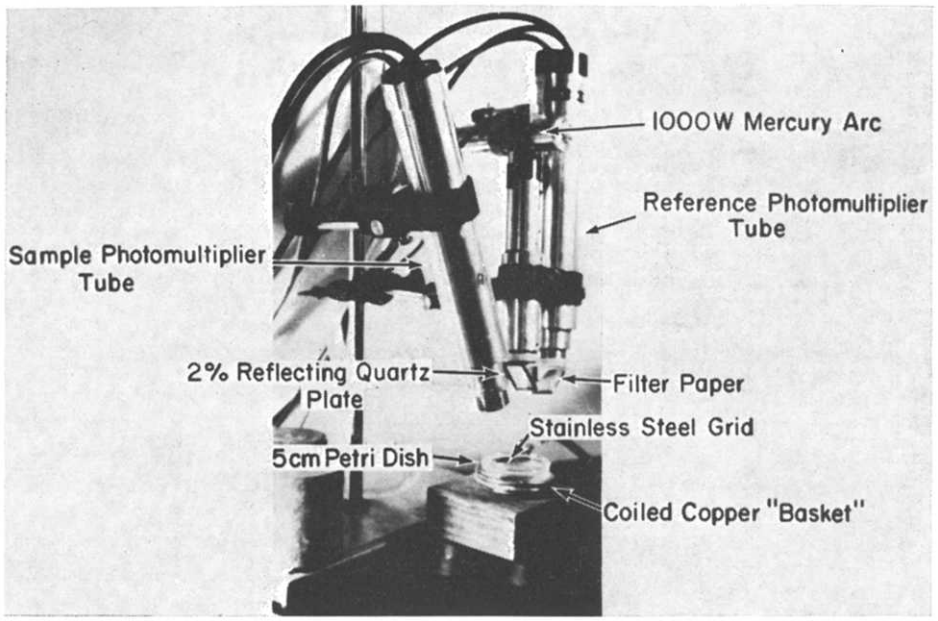


Fig. 1. Experimental apparatus consisting of differential fluorimeter and thermostated culture dish.

fluorescence in the cultured organ to a jet of 95 % N_2–5 % CO_2 blown onto the surface of the tissue and by the ability of the tissues, on several occassions to be re-cultured in hormone-free medium for 2 days and used as experimental material a second time. Cultures which were used more than once produced acid and their pyridine nucleotide component was rapidly responsive to the anoxic effect of N_2 gas.

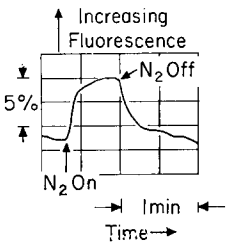
RESULTS

Fig. 2 shows the change in fluorescence which results from blowing N_2 gas across the surface of a prostate organ culture, thereby making it anaerobic and the effect of subsequently removing the N_2.

Fig. 3A shows that the addition of testosterone to cultures containing 0.1 % glucose is followed after 40 to 60 min by a gradual increase in the level of reduced pyridine nucleotide fluorescence. In 2 experiments, done on the same day, 3 cycles of increasing and decreasing fluorescence are superimposed upon the increasing steady-state fluorescence level. In these experiments the time lapse between the first addition of testosterone and a 1 % increase in fluorescence is 38.5 $\pm$ 3 min. After the oscillations have stopped there is an average net increase in fluorescence of 8.5 $\pm$ 0.5 %. Table I shows that the period and amplitude of the oscillations increases slightly from the first to the third. These cycles of pyridine nucleotide reduction–oxidation are unlike those seen in yeast[4,5] for they stop just as they become maximal, while in yeast the amplitude decreases exponentially while the period is constant. The glucose level is quite critical in the production of oscillations. When 0.05 % glucose is used rather than 0.1 %, a single addition of testosterone causes only a 2 % increase in the basal fluorescence, and no oscillations are seen (Fig. 3B). Experiments done in the

0.05 % glucose medium showed that 53 min elapsed between the time when a single addition of testosterone was made and when the fluorescence increased 1 %. The maximum increase in fluorescence recorded was 2.3 %.

Fig. 4A shows that when glucose additions were made to a prostate culture in 0.05 % glucose after testosterone treatment there was a further increase in reduced pyridine nucleotide fluorescence totalling about 16 %. The responses to glucose addition become smaller with time as the enzymatic sequence involved in reducing pyridine nucleotide apparently becomes saturated. Fig. 4B shows a control experiment in which a glucose addition was made to a prostate culture before testosterone addition. It can be seen that quenching of fluorescence occurred.



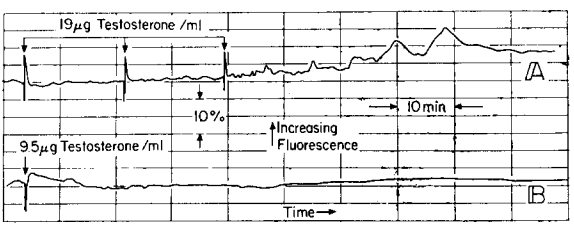


Fig. 2. N_2–air cycle of pyridine nucleotide reduction and oxidation obtained by blowing a stream of 95 % N_2–5 % CO_2 onto the surface of a prostate organ culture.

Fig. 3. A. Kinetics of increased reduced pyridine nucleotide fluorescence in cultured rat prostate in response to three 19 μg/ml additions of testosterone. The medium in which the culture was incubated contained 0.1 % glucose. B. Kinetics of pyridine nucleotide reduction in response to one 9.5 μg/ml testosterone addition when the culture is maintained in Eagles medium containing 0.05 % glucose.

TABLE I

AMPLITUDE–PERIOD CHARACTERISTICS OF PYRIDINE NUCLEOTIDE OSCILLATIONS IN ORGAN CULTURES OF RAT PROSTATE

	Oscillation number		
Amplitude (per cent fluorescence)	2.75	4.25	4.75
Period of oscillation (min)	4.15	4.25	4.75

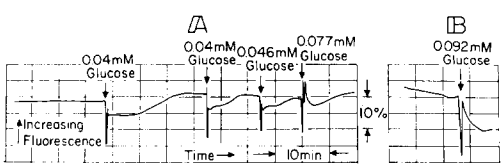


Fig. 4. A. Rapid primary fluorescence quenching and secondary fluorescence stimulation following glucose addition to a testosterone-pretreated prostate culture. The culture which was used is the same as that from which Fig. 3B was obtained. The experiment shown in this figure was done immediately after the experiment shown in Fig. 3B. The glucose concentrations which are shown indicate amounts above the basal 0.05 % level present in the medium. B. The primary fluorescence quenching effect is the only one seen when glucose is added to a prostate culture which had not treated with testosterone.

Hydrocortisone addition to a prostate culture produced a rapid cycle of reduced pyridine nucleotide fluorescence (Fig. 5). The response begins within 2 to 5 min, attains a maximum after 5 to 12 min and is complete after 20 to 30 min. Fig. 5 also shows that if testosterone is added to the prostate culture after the response to hydrocortisone is complete, the reduced pyridine nucleotide fluorescence begins to increase within 2 to 3 min; the 35 min lag is not seen.

Fig. 6 shows the effect of adding 30 μl of 50 % ethanol, the vehicle for the steroid hormones, to a prostate culture. The ethanol produces no significant change in the prostatic fluorescence. Fig. 5 also shows the effect of adding 17β-estradiol to a prostate culture previously treated with hydrocortisone and testosterone. Each of 3 additions

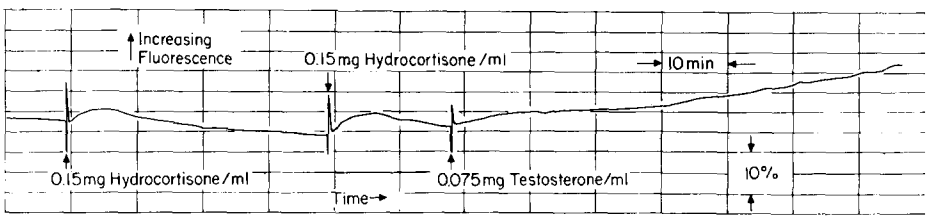


Fig. 5. Kinetics of reduced pyridine nucleotide fluorescence in response to 2 sequential additions of hydrocortisone (0.15 mg/ml) and to the addition of testosterone (0.075 mg/ml) after the effect of hydrocortisone was complete.

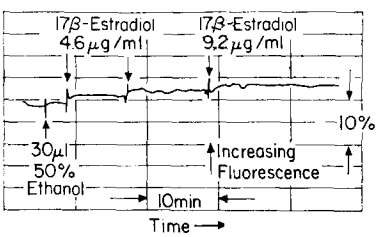


Fig. 6. Fluorescence traces showing the effect of adding ethanol, the vehicle in which the steroid hormones were dissolved or suspended, and the effect of adding 17β-estradiol.

of estradiol increased the tissue fluorescence between 1 and 2 %. The effect was essentially instantaneous, and slower changes indicating estrogen influence upon the redox state of intracellular pyridine nucleotide were not seen.

DISCUSSION

Testosterone addition to cultures incubated in 0.1 % glucose is followed by an increase in reduced pyridine nucleotide fluorescence. This seems to be the result of an increase in the state of cytoplasmic reduction following an increase in the rate and amount of glycolysis, for it proceeds through a series of oscillations similar to those which occur when glucose is added to starved yeast cells[4,5]. The testosterone has apparently allowed glucose free access to the existing glycolytic sequence[6]. Oscillations of glycolysis proceed only where pyridine and adenine nucleotides are shared by control sites in the glycolytic enzyme sequence. As soon as oxidative phosphorylation competes with glycolysis for these nucleotides, presumably the oscillations should stop[5]. It would appear that the sudden end of the oscillations after 3 have been completed

indicates that mitochondrial processes have begun to share nucleotides with glycolysis.

When glucose additions are made to a testosterone-treated prostate culture in 0.05 % glucose, incremental increases of reduced pyridine nucleotide fluorescence are also seen. This glucose-stimulated increase in fluorescence seems to become saturated after it has increased about 16 %. The 16 % increase in fluorescence is about the same as was seen when both hydrocortisone and testosterone were added to a culture in 0.1 % glucose.

It seems unlikely that the steroids are producing pyridine nucleotide reduction by inhibiting respiration, either directly or by a Crabtree effect. There does not seem to be sufficient pyridine nucleotide present before hormone addition to account for the large pyridine nucleotide reduction which is seen. A N_2–air cycle before hormone addition produces a fluorescence change of 4 %. However, the increase in reduced pyridine nucleotide fluorescence seen after testosterone was added to a culture in 0.1 % glucose was at least 8 %. Moreover, a direct inhibition of respiration would be expected to occur within several minutes, but the increase in reduced pyridine nucleotide fluorescence seen after testosterone addition took about 50 min, and that following glucose addition took about 15 min.

It appears that hydrocortisone and testosterone have synergystic effects upon prostatic reduced pyridine nucleotide fluorescence. The addition of testosterone to a culture after the hydrocortisone-stimulated redox cycle is complete is followed almost immediately by an increase in reduced pyridine nucleotide fluorescence. When testosterone was added alone, 35 to 50 min elapsed before the reduced pyridine nucleotide fluorescence increased significantly (about 8 %). But when hydrocortosone and testosterone were both added the steady-state fluorescence increased about 16 %.

N_2–air cycles indicate the amounts of both pyridine nucleotide linked to respiration and glycolytic pyridine nucleotide were increased after hormone addition. In an experiment like that shown in Fig. 5, the change in fluorescence resulting from a N_2–air cycle before hormones were added was 4 %. After both hydrocortisone and testosterone were added and the new steady-state was reached, a N_2–air cycle resulted in a fluorescence change of 9 %, indicating a doubling of pyridine nucleotide linked to respiration. But the steady-state fluorescence increased 15 %. This seems to indicate that 6 % of the fluorescence increase was due to additional glycolytic pyridine nucleotide.

The compensated tissue fluorimeter (Fig. 1) is designed so that a narrow band of near ultraviolet light isolated by a heat filter and a Corning 18A filter and having a maximum of 366 mμ is focused onto the tissue. Fluorescence emission from the tissue, mixed with reflected 366 mμ light is collected by a lens, filtered by Corning 47 and Wratten 2A filters to eliminate all radiation but the reduced pyridine nucleotide fluorescence emission and focused onto a photomultiplier tube. The fluorescence excitation and emission bands of reduced pyridine nucleotide are about 100 mμ shorter than those of the porphyrins, one group of substances which might be expected to interfere. A second group of substances which are present and which fluoresce are the steroids. Of these the most fluorescent is 17β-estradiol[8,9]. Androgens and corticosteroids are non-fluorescent. However, the absorption maximum of estradiol is 285 mμ, and the maximum emitted fluorescence is 330–340 mμ. These considerations make it unlikely that significant native steroid fluorescence would

interfere with the determination of reduced pyridine nucleotide. However, in Fig. 6 it can be seen that some estrogen fluorescence is stimulated by and detected with the compensated fluorimeter.

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