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THE EFFECT OF METYRAPONE ON CELLULAR RESPIRATION AND MICROSOMAL DRUG OXIDATION*

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Abstract—Five mM metyrapone increases the lactate–pyruvate ratio in the perfusion fluid of perfused rat livers by a factor of 9 and in rat liver slices by a factor of 3. Furthermore, it decreases the rate of oxygen uptake of liver slices by 50 per cent. The reduced metabolite of metyrapone also inhibits respiration of liver slices. From these results evidence is taken for an inhibitory effect of metyrapone and its main metabolite on the respiratory chain leading to a change of the redox status of the cell and to an increase of aerobic glycolysis.

In mouse liver microsomes the rate of oxygen consumption in the absence of exogenous substrates is not affected by 2×10^{-4} M metyrapone, although metyrapone in this concentration already inhibits microsomal drug hydroxylation. When the inhibitor SKF 525-A is added oxygen uptake increases, thereby confirming the nature of the inhibitor as a substrate of microsomal hydroxylases. This increase can be diminished by the subsequent addition of 2×10^{-4} M metyrapone. These results indicate that metyrapone as an inhibitor of drug metabolism acts on the binding of substrates to cytochrome P-450 rather than on microsomal electron transport.

METYRAPONE [Su 4885, Metopirone®, 2-methyl-1,2-bis(3′-pyridyl)1-propanone] is known to inhibit various adrenal mitochondrial steroid hydroxylases.[1, 2] Steroid hydroxylases are very similar to the drug metabolizing enzyme systems in liver microsomes. For this reason steroids have been suggested to be the physiological substrates of drug hydroxylating liver enzymes.[3] In both types of hydroxylases the incorporation of oxygen is mediated by the same hemoprotein, cytochrome P-450, as the terminal oxidase.[4, 5] From these similarities it could be expected that metyrapone would also interfere with drug hydroxylation, which it does: it has been shown both *in vivo* and *in vitro* to inhibit the demethylation of amidopyrine and *p*-nitroanisole as well as the ring hydroxylation of acetanilide.[6, 7]

While studying the metabolic transformation of metyrapone itself in the isolated perfused rat liver we observed that this substance acts on cellular metabolism in a more general way. The experiments described in this paper show that metyrapone, besides its action on microsomal drug metabolizing enzymes, also inhibits the mitochondrial respiratory chain.

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MATERIALS AND METHODS

Perfusion experiments

For perfusion experiments male Sprague–Dawley rats of 250–300 g body weight were used. They were kept on a standard laboratory diet (Altromin®) and water *ad lib.* The perfusion procedure employed was largely that described by Miller *et al.*[8] and Schimassek,[9] however, livers were not removed from the rat but left *in situ* as described by Hems *et al.*[10] The rats were anesthetized with 6 ml/kg of a 20% (w/v) urethane solution. The perfusions were carried out at a constant pressure of 20 cm of water and with recirculating perfusion fluid. The perfusion fluid consisted of bovine erythrocytes washed three times and diluted to 40% (v/v) in Krebs–Ringer bicarbonate buffer containing 1·5 per cent (w/v) bovine serum albumin (Behring–Werke, Marburg) and 20 mM glucose. Following saturation with 5 per cent CO_2 in O_2, the pH of this medium was 7·4. Equilibration of the perfusion medium with the gas was achieved at 37° by having it pass over a thin film of fluid on the surface of a modified reflux condenser. Each perfusion experiment lasted about 2 to 3 hrs. Usually metyrapone was added to the perfusion medium 30 min after starting the perfusion. The volume of the perfusion fluid was about 100 ml.

Liver slice experiments

Rat liver slices were prepared in the cold room at 4° according to Stadie and Riggs.[11] They were incubated at 37° either in Krebs–Ringer phosphate buffer or in Krebs–Ringer bicarbonate buffer under an atmosphere of oxygen or 95 per cent oxygen and 5 per cent CO_2, respectively. Oxygen uptake was measured in a Warburg apparatus, the stopcocks of which were closed 30 min after the beginning of the incubation. In all cases control values of the rate of oxygen uptake were obtained for the next 30–45 min until the metyrapone or buffer solutions were tilted in from the side arms of the vessels. The inhibited and control respirations were measured for another 60–90 min. Thus each incubation served as its own control. In some experiments the influence of metyrapone on the respiratory rate of liver slices in Krebs–Ringer bicarbonate buffer under O_2/CO_2 was measured polarographically with the aid of a Clark platinum electrode.

In one experiment together with Dr. R. W. Estabrook the oxygen uptake of electron transport particles (ETP) derived from heart mitochondria[12] was measured polarographically[13] in the presence of metyrapone and amobarbital (Amytal®).

Lactate and pyruvate were estimated enzymatically in either the perfusion fluid or the slice incubation medium using the respective test kits of C. F. Boehringer a. Soehne, Mannheim, with some modifications. Samples of 2 ml of perfusion medium were deproteinized by adding 5 ml of ice cold 0·6 N perchloric acid. It was noticed that the calibration curve for pyruvate in contrast to the test instruction leaflet does not pass the ordinate at the zero point. This is due to the dilution of the originally present NADH (optical density about 1·8) by the addition of 0·05 ml of lactate dehydrogenase solution to 2·05 ml of total test solution in the cuvette. At 340 nm this deviation amounts to a decrease in optical density of about 0·040. The light absorption by metyrapone itself was accounted for.

Experiments with isolated microsomes

Liver microsomes of male general purpose mice of 20–30 g body weight were

prepared from pooled livers of about ten animals as described earlier for rat liver microsomes.[14] For stimulation of drug metabolizing activity the animals received four times 60 mg/kg of sodium phenobarbital i.p. within about 60 hr prior to their sacrifice.

The oxygen consumption of microsomal suspensions was recorded with the aid of a multi-wire platinum electrode (W. Eschweiler a. Co., Kiel) under constant magnetic stirring in 2·6 ml at 37°. The inhibitors were added with a micro syringe in a volume of 10 μl.

RESULTS

Liver perfusion experiments

When 5 mM metyrapone* is added to the perfusion fluid, the lactate pyruvate ratio shows a very marked increase. After 60 min of perfusion with the drug the ratio is elevated by a factor of about 9 above the respective control values without the inhibitor. In control experiments of equal or longer duration the lactate pyruvate ratio remained at a value of 10 during a period of up to 90 min and did not exceed a mean value of 15 in experiments which lasted up to 3 hr. According to various authors (cf. 15) these values constitute the normal range of lactate pyruvate ratios in perfused

TABLE 1. EFFECT OF METYRAPONE ON THE LACTATE PYRUVATE RATIO IN THE PERFUSION FLUID OF PERFUSED RAT LIVERS

		Minutes after addition of metyrapone		
	Controls	30	60	90
Lactate (μmoles/ml)	2·81 ± 0·85	6·46 ± 2·34†	7·62 ± 2·70†	10·10 ± 2·66†
Pyruvate (μmoles/ml)	0·32 ± 0·04	0·17 ± 0·05*	0·12 ± 0·06*	0·16 ± 0·04*
Lactate/pyruvate ratio	9·7 ± 4·4	41·3 ± 19·4†	84·8 ± 33·0†	68·5 ± 22·2†

Five mM metyrapone was added to the perfusion fluid usually 30 min after the beginning of the experiment. Samples were taken by collecting the outflowing perfusion fluid from the catheter in the vena cava inferior. Control samples were taken 5 min before adding metyrapone to the "blood" pool. Number of control experiments: 11, number of metyrapone experiments: 5. Values are means ± S.D.

Significance against respective controls: * $P < 0{\cdot}01$ † $P < 0{\cdot}001$.

livers. Table 1 shows that the elevation of the lactate pyruvate ratio is largely due to an increase of the lactate concentrations rather than a decrease in pyruvate.

Metyrapone has no influence on the perfusion rate, which only slightly decreases throughout the experiment. Also an increased production of lactate by the erythrocytes of the perfusion medium has been excluded by a simulated perfusion experiment with recirculating medium only, to which metyrapone was added. Furthermore, the enzymatic determination of lactate and of pyruvate is not influenced by metyrapone or its main metabolite (Su 5236),* in which the keto group has been reduced to a secondary alcohol group,[10] even in concentrations up to 10^{-2} M.

Experiments with liver slices

In liver slices the lactate pyruvate ratio was also increased by the addition of 5 mM

* Su 4885 and Su 5236 were kindly donated by Dr. J. J. Chart of CIBA Pharmaceutical Company, Summit, N.J., U.S.A.

metyrapone. In general, it is elevated by a factor of 3. Figure 1 shows the lactate and pyruvate concentrations in the incubation medium after 2 hr and reveals that the lactate pyruvate ratios are smaller than those in the perfusion experiments. The figure shows that similar to the perfusion experiments the elevation of the lactate pyruvate ratio is caused by an increase in lactate production and not so much by a decrease in pyruvate. The increase in lactate concentration by metyrapone can only be demonstrated in Krebs–Ringer bicarbonate buffer. In the corresponding phosphate buffer

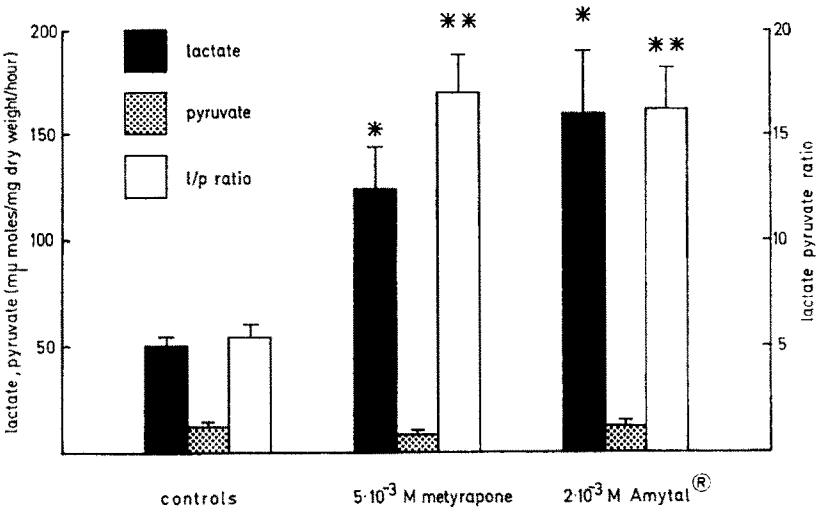

FIG. 1. Effect of metyrapone and Amytal® on lactate and pyruvate production and on the lactate pyruvate ratio of rat liver slices.

About 15 mg (dry weight) of slices were incubated for 80–150 min in 2·3 ml of Krebs–Ringer bicarbonate buffer pH 7·4, 37°, 95% O_2/5% CO_2. Mean values ± S.E.M. of six experiments each. * $P < 0.01$, ** $P < 0.001$.

we observed a smaller lactate production (33 ± 12 mμmoles of lactate per mg of dry weight per hour) and this was not increased significantly by metyrapone.

In Fig. 1 there are additional corresponding experiments with the almost classical inhibitor of mitochondrial respiration, Amytal®,[17] which increases the lactate pyruvate ratio.[18] From our results Amytal® is about twice as potent as metyrapone.

The observation of an apparently increased aerobic glycolysis in the presence of metyrapone suggested that the primary action of this inhibitor consists in an inhibition of cellular respiration.

We studied, therefore, the oxygen uptake of liver slices in both Krebs–Ringer phosphate and bicarbonate buffers manometrically and polarographically, respectively, and found that metyrapone reduces the oxygen consumption by about 50 per cent. The metyrapone concentration in these experiments was identical to that which was previously shown to elevate the lactate pyruvate ratio. This is demonstrated in Fig. 2. It also shows that the reduced metabolite of metyrapone (Su 5236) exerts about the same effect on the respiratory chain. However, both compounds are about half as effective as Amytal®.

In bicarbonate buffer the polarographically measured rate of oxygen consumption (0·27 ± 0·05 μmoles O_2 per mg dry weight per hour; mean ± S.D.; n = 5) corresponds closely to the manometrically measured rate in phosphate buffer (0·24 ± 0·06 μmoles O_2 per mg dry weight per hour; mean ± S.D.; n = 34). The inhibitory effect of metyrapone on oxygen uptake in bicarbonate buffer is identical to that in phosphate buffer.

It may be added here that the polarographic recording of the rate of oxygen consumption by electron transport particles (ETP) yielded the same results regarding the effects of metyrapone and Amytal®.[19] The respective concentrations for 50 per cent

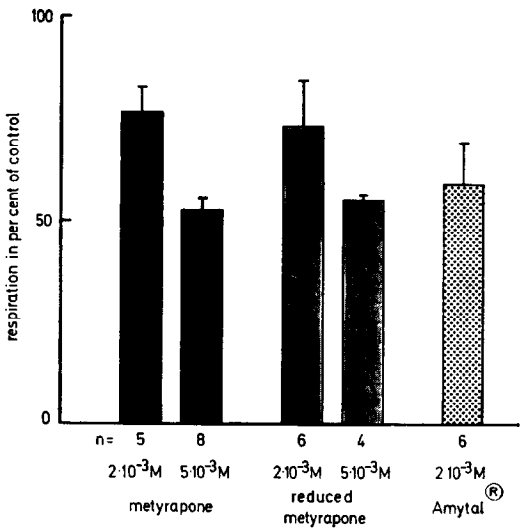

FIG. 2. Inhibitory effect of metyrapone and reduced metyrapone on respiration of rat liver slices in comparison to that of Amytal®.

About 15 mg (dry weight) of rat liver slices were incubated in a Warburg apparatus for 80–150 min in 2·3 ml of Krebs–Ringer phosphate buffer pH 7·4, 37°, under an atmosphere of oxygen. Values are means ± S.D. in per cent of control respiration periods in the same vessel before addition of the inhibitors from the side arm. Percentage of inhibition was corrected for a 5 per cent decrease of oxygen uptake in respective blank experiments, in which buffer solution was added instead of the inhibitors. $P < 0{\cdot}01$. The absolute mean value ± S.D. of all control periods was 0·24 ± 0·06 μ moles O_2/mg dry weight per hour, n = 34.

inhibition were 1·5 mM for metyrapone and 0·6 mM for Amytal®. Respiration in this experiment was initiated by the addition of NADH.

Experiments with mouse liver microsomes

Since metyrapone was shown to inhibit total cellular respiration, the question arose whether this agent can also reduce the oxygen uptake of isolated drug metabolizing microsomes. We have, therefore, recorded the oxygen uptake of phenobarbital stimulated mouse liver microsomes using a platinum electrode (Fig. 3). The tracings show that 2×10^{-4} M metyrapone does not affect the course of oxygen disappearance when no exogenous substrate is present (Fig. 3, curve A). This distinguishes metyrapone

clearly from another microsomal inhibitor, SKF 525-A. In contrast to metyrapone the addition of SKF 525-A leads to an increase in oxygen consumption (Fig. 3, curve B) which possibly represents the oxidative dealkylation of SKF 525-A itself. Therefore, SKF 525-A must be considered as a substrate of microsomal hydroxylases, which agrees with the demonstration of dealkylated products by Anders *et al.*[20] Figure 3 demonstrates that upon addition of 2×10^{-4} M metyrapone the increased rate of

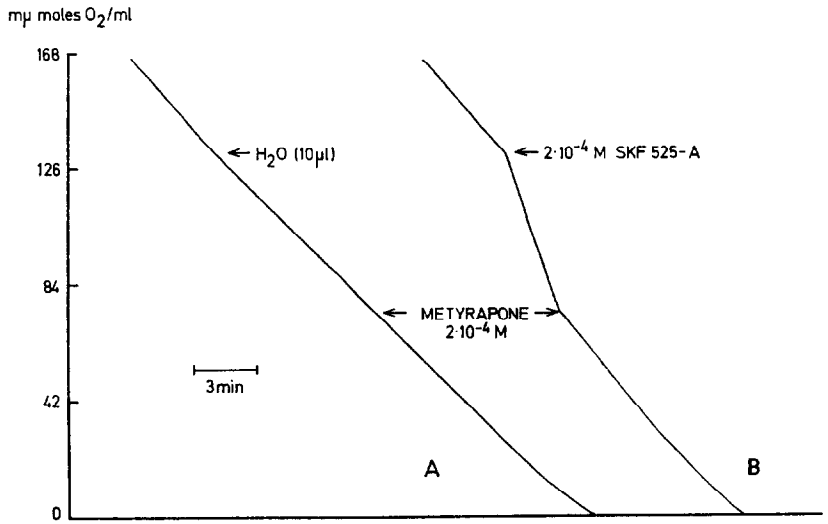

FIG. 3. Effect of metyrapone on microsomal oxygen uptake. Mouse liver microsomes from phenobarbital stimulated animals, 0·35 mg protein/ml, were incubated in phosphate buffer pH 7·8 at 37° in the presence of nicotinamide and a NADPH regenerating system according to Netter.[14] Respiration rate was measured polarographically in 2·6 ml. Substances were added in a volume of 10 μl of water.

oxygen consumption, which was induced by SKF 525-A, disappears. This result suggests that metyrapone interferes with the microsomal respiration only when a hydroxylable substrate is present. The basic oxygen consumption of microsomes is not significantly affected.

The applied concentration of metyrapone corresponds to that previously found to inhibit the oxidative conversion of *p*-nitroanisole to *p*-nitrophenol by about 50 per cent.[21] Thus the experiment once more manifests the substrate properties of SKF 525-A.

DISCUSSION

The steroid hydroxylase inhibitor metyrapone reduces the oxygen uptake of liver tissue and impairs the hydroxylation of foreign compounds. Concomitantly the formation of lactate and the lactate pyruvate ratio increase several fold.

Increases of the lactate pyruvate ratio are observed in a number of conditions e.g. starvation,[22] diabetes mellitus,[23] hypoxia,[24] and application of drugs such as Amytal®[18] or ethanol.[25] Generally such increases are taken as evidence for a change of the ratio of free NAD to free NADH in favour of the reduced pyridine nucleotide.[26]

Since metabolic regulations cannot be considered as intact in tissue slices,[27] we have calculated the NAD–NADH ratios in the cytosol from the data obtained by liver perfusion. Using the newly determined equilibrium constant for lactic dehydrogenase[28] our results indicate that 60 min of perfusion with metyrapone will decrease the NAD–NADH ratio from about 929 to 106. Accordingly the redox potential of the lactate pyruvate system changes from −234 mV to −263 mV, based on a value of −204 mV for E'_0 (pH 7·0, 37°).

It can be concluded that the primary action of metyrapone, which leads to the described change in the redox status of the cells, consists in a partial blocking of the mitochondrial electron transport. This view is supported by the decreased oxygen uptake in liver slices. Furthermore, metyrapone has been shown to inhibit respiration in isolated rat liver mitochondria[29] and in electron transport particles from heart mitochondria.[19] In these experiments metyrapone produced the same effects as did Amytal®, whereby equi-effective concentrations for metyrapone are twice as high as those for Amytal®.

Besides the described effect, metyrapone may have other actions on cell metabolism. Thus an inhibition of β-hydroxybutyrate dehydrogenase, which has been discussed,[30] would interfere with the transport of hydrogen from the cytoplasm into the mitochondria.

Having recognized its action on mitochondria, we asked, whether metyrapone inhibits the drug oxidation by interfering with the microsomal electron transport. A comparison of the inhibitory concentrations reveals that the microsomal hydroxylases are far more sensitive than the respiratory chain. A 50 per cent inhibition of mitochondrial respiration requires about 5×10^{-3} M metyrapone, while a similar reduction of *p*-nitroanisole demethylation is already achieved at about 2×10^{-4} M.[21] In microsomes in the absence of exogenous substrates metyrapone does not influence the basal oxygen uptake. This agrees well with the previous observation that metyrapone does not affect the microsomal NADPH oxidation in the presence of the substrate *p*-nitroanisole.[6]

These results seem to exclude a direct interaction of metyrapone at the level of electron transport to cytochrome P-450. This assumption is supported by the previous observation[21] that metyrapone actually is bound to cytochrome P-450 producing spectral changes characteristic of type II binding (aniline) of a substrate.[31] Furthermore, it was shown by kinetic experiments that metyrapone inhibits the demethylation of *p*-nitroanisole competitively,[21] thereby also suggesting an interaction at the binding site for substrates.

Metyrapone itself apparently is not a substrate readily hydroxylated by the drug metabolizing enzymes as is seen from its failure to stimulate microsomal oxygen uptake.

In contrast to metyrapone the inhibitor SKF 525-A shows a different behaviour in that it rather stimulates the microsomal oxygen consumption in concentrations, which actually decrease the hydroxylation rate of drugs. At present this effect is explained by the fact that SKF 525-A itself is a substrate which is oxidatively dealkylated.

Recent experiments[32] show that the reoxidation of anaerobic cytochrome P-450, which was reduced by NADPH, exhibits different spectral characteristics in its time course depending on the presence or absence of substrate (amidopyrine). Metyrapone

is an inhibitor which is bound to cytochrome P-450[21] but apparently not hydroxylated to a large extent. In this way, perhaps, the unique inhibitor could prove itself to be a valuable tool in the further elucidation of microsomal oxidation reactions.

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