

**Metabolism of 7-Benzoyloxy- 7-Ethoxy- and 7-Pentoxo-resorufins by liver
and olfactory microsomes from Rats and Guinea Pigs**

Effect of adding dicumarol *in vitro*

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Abstract

The O-dealkylations of 7-alkoxy-resorufins are very widely studied Cytochrome P450 (CYP)-dependent activities, as resorufin, a highly fluorescent compound is produced and this allows the reaction to be followed in a spectrophotofluorimeter. Relatively crude tissue preparations can be used as the enzyme source, such as the 9000 g supernatant of the homogenate, provided that dicumarol, an inhibitor of DT-diaphorase (NAD(P)H:quinone oxidoreductase 1), is added to prevent further reduction of resorufin to non-fluorescent products. The purpose of this communication is to draw attention to a unexpected interaction of dicumarol with the microsomal metabolism of 7-alkoxy-resorufins. We report that dicumarol, added to liver microsomal suspensions in concentrations known to inhibit DT-diaphorase-mediated reduction of resorufin, will stimulate markedly the microsomal metabolism of 7-benzyloxy- and 7-pentoxy-resorufin by liver microsomes of male rats. The effect is sex-, tissue- and species-specific; no such stimulation is seen with liver microsomes from female rats, neither it is observed with olfactory microsomes from male rats or with liver microsomes from guinea pigs of either sex. It is also substrate-specific, in the sense that the metabolism by liver microsomes of male rats of another 7-alkoxy-resorufin, 7-ethoxy-resorufin, is not stimulated by addition of dicumarol. The results are consistent with dicumarol exerting “ heterotropic cooperativity” in the 7-benzyloxy- (and 7-pentoxy-) resorufin metabolism by a constitutive male-specific member of the CYP3A subfamily (possibly CYP3A2), although some more work is necessary to provide conclusive evidence for this interpretation.

Keywords: 7-alkoxyresorufins, olfactory epithelium, liver microsomes, dicumarol.

INTRODUCTION

The O-dealkylations of 7-alkoxy-resorufins are very widely studied Cytochrome P450 (CYP)-dependent activities, as resorufin, a highly fluorescent compound is produced and this allows the reaction to be followed in a spectrophotofluorimeter (1). Relatively crude tissue preparations can be used as the enzyme source, such as the 9000 g supernatant of the homogenate, provided that dicumarol, an inhibitor of DT-diaphorase (NAD(P)H:quinone oxidoreductase 1), is added to prevent further reduction of resorufin to non-fluorescent products (2). The main purpose of this communication is to draw attention to unexpected interactions of dicumarol with the microsomal metabolism of 7-alkoxy-resorufins. We report that dicumarol, added to liver microsomal suspensions in concentrations known to inhibit DT-diaphorase-mediated reduction of resorufin (2), will stimulate markedly the metabolism of 7-benzyloxy- and 7-pentoxy-resorufin by liver microsomes of male rats. The effect is sex-, tissue- and species-specific; no such stimulation is seen with liver microsomes from female rats, neither it is observed with olfactory microsomes from male rats or with microsomes from guinea pigs of either sex. It is also substrate-specific, in the sense that the metabolism by liver microsomes of male rats of another 7-alkoxy-resorufin, 7-ethoxy-resorufin, is not stimulated by addition of dicumarol. The results are consistent with dicumarol exerting “heterotropic cooperativity” in the 7-benzyloxy- (and 7-pentoxy-) resorufin metabolism by a constitutive male-specific member of the CYP3A subfamily (possibly CYP3A2), although some more work is necessary to provide conclusive evidence for this interpretation.

Materials and methods

Chemicals

NADPH and dicumarol were from Sigma Chemical Co. (Poole, U.K.). Resorufin, 7-benzyloxy-, 7-pentoxy- and 7-ethoxy-resorufin were from Boehringer Corp. (Lewes, U.K.).

Assays

Tissue protein concentrations were determined according to Lowry *et al.* (3), using bovine serum albumin as a standard. The activities of the 7-alkoxy-O-dealkylases were measured, using either a 9000 g, postmitochondrial fraction or isolated microsomes, essentially as described by Lubet (2). The incubation mixture contained the following components in a total volume of 2.25 mL: 20-200 microL of enzyme solution, 10 µL of an alkoxyresorufin solution in DMSO, 2 mL of 50 mM Tris-HCl buffer, pH 7.5 (containing 25 mM MgCl₂) and, where indicated, dicumarol to a final concentration of 20 µM (2). The final concentrations of the 7-ethoxy-, 7-pentoxy- and 7-benzyloxy-resorufins were 2,10 and 5 µM, respectively. The reaction was started by addition of NADPH (125 µM) and the production of resorufin followed by the increase in fluorescence at 37 ° C, using an excitation wavelength of 522 nm and an emission wavelength of 586 nm.

Animals and tissue preparations

Male and female rats (150-200 g body wt) of the LAC:P Wistar-derived strain and guinea pigs of either sex and male guinea pigs (240-470 g body wt) of the Hartley strain were used. They were allowed food and water *ad lib.* or their food withdrawn overnight before being killed at 9.00-10.30 a.m. All procedures involving animal care were in agreement with national and international laws and policies.

The liver and olfactory tissue homogenates were prepared and centrifuged to obtain the corresponding post-mitochondrial, 9000 g supernatants, microsomal pellets and cytosolic fractions, as described by Manno *et al.*, (4) and by Reed *et al.* (5).

RESULTS AND DISCUSSION

Profile of 7-alkoxyresorufin-O-dealkylases in the liver and olfactory epithelium of rats and guinea pigs.

The profile of dealkylases activities found in the liver of untreated rats and guinea pigs was found to be significant different in the two species. In the text that follows the O-dealkylations of 7-benzyloxy-, 7-ethoxy- and 7-pentoxo-resorufin are abbreviated as BROD, EROD and PROD, respectively. When measured in isolated microsomes in the absence of dicumarol [see Figure 1, panels A and B], the activity of BROD clearly predominated over the other two dealkylases in the liver of the guinea pig, while in rat liver the most active dealkylase tended to be EROD. In the olfactory epithelium EROD was the most active enzyme in both species, although, also in this tissue, the BROD activity was significantly greater in the guinea pig.

There were a significant sex differences in the hepatic BROD and PROD activities of the rats (greater in males: $P < 0.01$ for both) and in the hepatic EROD activity of guinea pigs (greater in the female: $P < 0.01$).

The effect of fasting was only studied in rats. No significant changes were observed in the female rats, but an increased liver BROD and PROD activity, compared to fed animals, was found in the male rat: $P < 0.001$ and $P < 0.01$, respectively.

In contrast to these sex and nutritional differences in liver enzymes, no such changes were found in the dealkylase activities of the olfactory epithelium.

Effect of dicumarol on the 7-alkoxy-resorufin-O-dealkylases in microsomal preparation from liver and olfactory epithelium of rats and guinea pigs.

When dicumarol was incorporated in the microsomal incubation [see Figure 1, panels C and D] at the concentration suggested for the assay of these dealkylases in the 9000 g supernatant fraction (2), all three dealkylases tended to be inhibited by dicumarol in both tissues of female rats and in those of guinea pigs of both sexes, the inhibition being more pronounced in the case of the guinea pig. In contrast, the activities of BROD and PROD were markedly stimulated in the liver of the male rat, both in its fed ($P<0.01$ and $P<0.05$) and fasted status ($P<0.01$ for both enzymes).

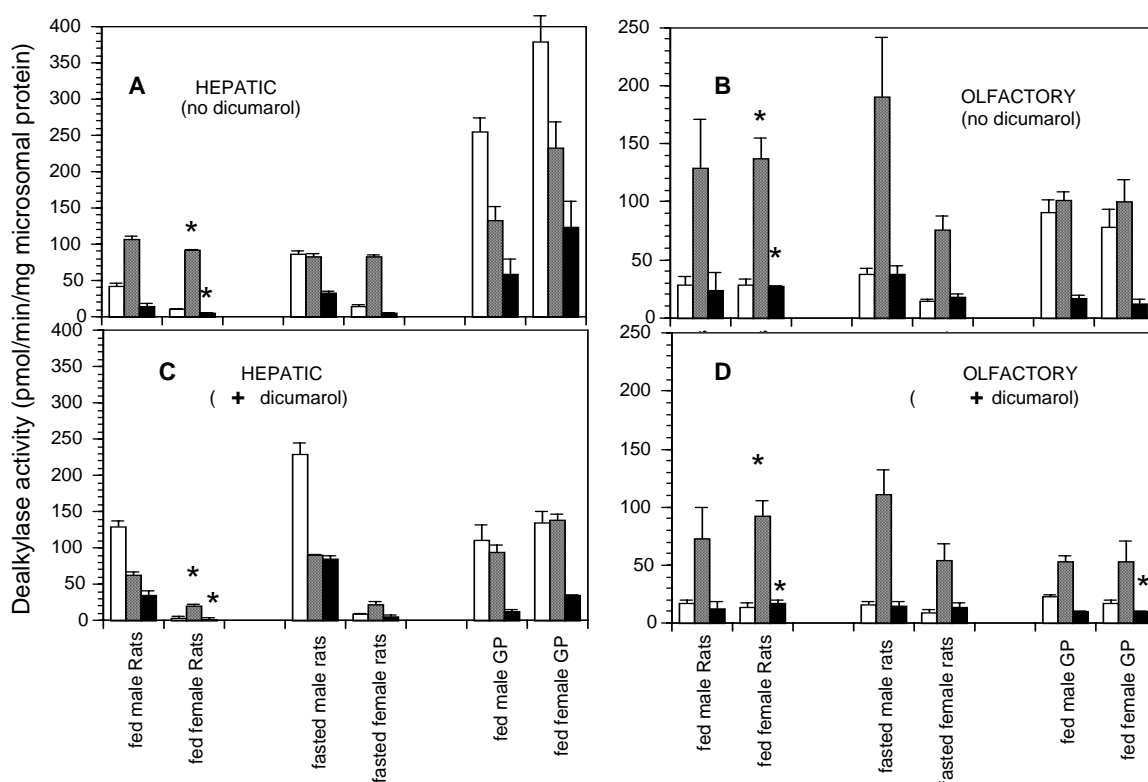


Figure 1

Microsomal 7-alkoxy-O-dealkylase activities in liver and olfactory epithelium of untreated rats and guinea pigs of both sexes. Rats were also examined after an overnight fasting. The activities were assayed both in the absence of dicumarol (panels A and B) and in its presence (panels C and D). Enzymes are shown in this order: BROD (white column); EROD (dark grey); PROD (black). Results are means \pm SEM of 3-6 observations. Results marked thus * are means of two observations with scatter.

This response was therefore sex-, tissue- and species-specific [Figure 2], suggesting the presence of a factor which makes only the liver of the male rat susceptible to stimulation. It was also substrate-specific, in the sense that the metabolism by liver microsomes of male rats of another 7-alkoxy-resorufin, 7-ethoxy-resorufin, was not stimulated by addition of dicumarol. As discussed below, this dicumarol stimulation may depend on a male specific CYP enzyme.

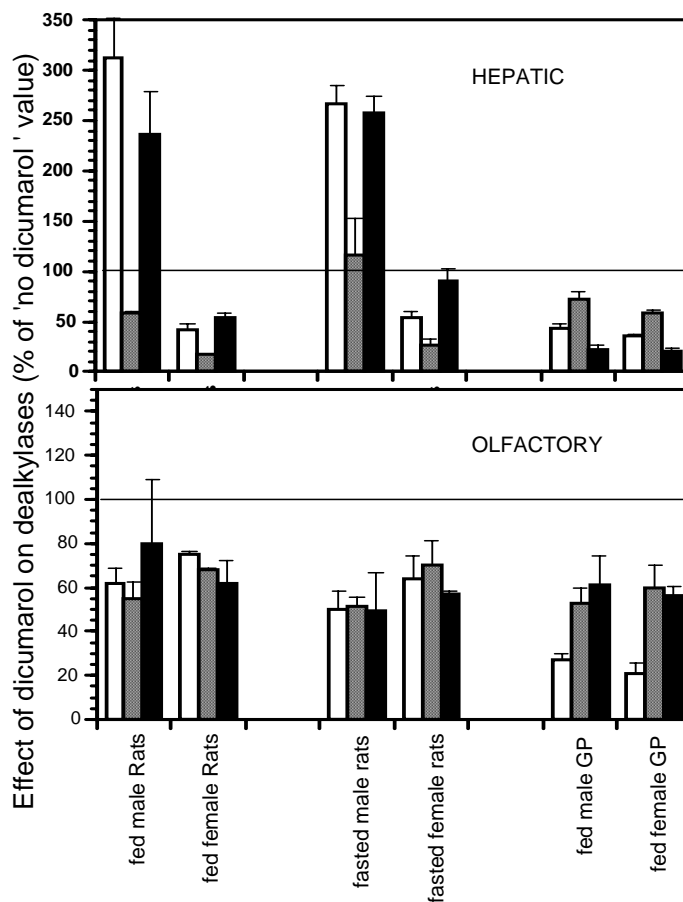


Figure 2

Effect of adding dicumarol on the 7-alkoxy-O-dealkylase activities of microsomes from liver and olfactory epithelium of untreated rats and guinea pigs of both sexes. The activities in presence of dicumarol are shown as a percentage of those obtained in the same samples incubated without dicumarol, the 100 % line drawn across the figures: BROD (white column); EROD (dark grey); PROD (black). Results are means \pm SEM of the number of observations given for Figure 1.

Since the addition of dicumarol to microsomes from male rats affected the three dealkylases differentially, the ratios between the three activities observed in the same microsomal preparation was markedly changed by addition of dicumarol. When the activities of PROD and BROD were expressed as a percentage of the EROD activity of the same sample (the latter was the least affected by dicumarol addition), a marked and highly significant increase was found for both PROD and BROD, compared to the corresponding percentage values seen in the absence of dicumarol (Table 1).

Table 1. Ratios between the activities of the 7-alkoxy-resorufin-O-dealkylases in liver microsomal fractions from fed male rats, with and without the addition of dicumarol. Comparison with the activities observed in the presence of dicumarol in the 9000 g liver supernatants obtained from rats of the same sex and nutritional state. The BROD and PROD activities are all expressed as a percentage of the EROD activity of the same sample, and are given as averages with SEM of the number of observations in parenthesis, or as individual observations. * $P < 0.001$; ** $P < 0.01$, compared to corresponding values obtained in the absence of dicumarol.

Liver preparation	Dicumarol	BROD activity	PROD activity
		(% of corresponding EROD activity of the same sample)	
A]			
<u>Isolated</u>	absent	40 ± 5 (4)	14 ± 1.4 (4)
<u>microsomes</u>	present	209 ± 13.6 (4)*	68 ± 10.7 (4)**
B]			
<u>9000 g supernatants</u>			
sample 1	present	250	59.5
sample 2	present	263	65

The values of BROD and PROD, again expressed as a percentage of the corresponding EROD values, were also markedly increased in the 9000g supernatants from livers of male rats (with values very close to those of the microsomal activities measured in presence of dicumarol), strongly suggesting that

dicumarol stimulates these two dealkylases also in the 9000g supernatant from the liver of the male rat. In one additional experiment the BROD and PROD activities of microsomes from a male rat were measured in presence of dicumarol, comparing a) the isolated microsomes to b) the same microsomes resuspended in their cytosolic fraction; very similar activities were found for the two preparations (results not shown).

The dicumarol-dependent stimulation of BROD and PROD may be an example of “heterotropic cooperativity” (6) with respect to a male-specific member of the CYP3A subfamily (probably CYP3A2 – which is known to be constitutively expressed as a male-specific enzyme in rat liver (7). This would require the enzyme to display mainly BROD, but also PROD activity, and to exhibit a stimulatory response to dicumarol, something which can be verified using the pure, genetically expressed, recombinant CYP enzyme. There is in fact evidence that 7-pentoxy-resorufin and 7-benzyloxy-resorufin are metabolized by more than one CYP enzyme, including CYP3A (8,9). 7-Benzyloxy-resorufin is a relatively non-selective substrate used in rats to monitor induction of CYP1A, CYP2B and CYP3A, but has also been suggested as a selective indicator of CYP3A activity (10,11).

The alternative explanation that the stimulation is related to the inhibition by dicumarol of DT-diaphorase would again require a species-, sex- and tissue-selective effect and, within the liver of the male rat, a discrimination between the three dealkylases, which we view as unlikely. Also, when the dealkylase activities of the hepatocytes from a male rat were measured in presence of dicumarol, comparing a) the isolated microsomes to b) the same microsomes resuspended in their cytosolic fraction (where most of the DT-diaphorase is located) very similar activities were found for the two preparations (see above), again suggesting that inhibition of DT-diaphorase is not the explanation for the dicumarol-mediated effects we have described.

GENERAL CONCLUSION

Dicumarol has been found to produce unexpected interactions with the 7-alkoxy-resorufin dealkylases in the liver and olfactory microsomes, inhibiting them in most cases. However, with the hepatic BROD and PROD activities of the male rat, dicumarol produced a marked stimulation, suggesting a species-, sex- and tissue-specific response, possibly an example of “heterotropic cooperativity” exerted by dicumarol on a male-specific constitutive CYP enzyme of the rat. The male-specific stimulation by dicumarol of BROD and PROD activities is also detected in presence of cytosol and will probably apply to the common assay of these dealkylases, when – that is - the 9000 g supernatant is used as the source of the enzyme. These interactions must therefore be born in mind when measuring the activities of these dealkylases both under basal conditions and after treatment with CYP inducers or inhibitors.

Aknowledgement

The Authors would like to thank the Medical Research Council of Great Britain for support and Carolyn Harvey for skilled technical assistance. F. De Matteis would also like to thank The Royal Society of Edinburgh for an Auber Awar .

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