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Effects of phenacetin on respiration in mitochondria isolated from rat kidney, liver and brain

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It is now virtually beyond dispute that prolonged or excessive consumption of analgesic preparations containing phenacetin (4-ethoxyacetanilide) can lead to serious kidney disease (for review see [1]). Although the reasons for this phenacetin-related nephropathy are not well understood it appears likely that a depression of renal respiratory activity is involved [1, 2], but whether this is due to a direct inhibition of the respiratory mechanisms of the cells or is exerted indirectly through causing, for instance, local methaemoglobinaemia or ischaemia, is uncertain. However, in earlier studies with *in vitro* preparations of rat kidney we found that phenacetin inhibited succinate dehydrogenase and partly blocked the mitochondrial electron transport chain between NADH dehydrogenase and Coenzyme Q, consequently inhibiting respiration in intact kidney cells [3]. Such actions *in vivo* would almost certainly bring about tissue damage and impair renal function since the kidney relies heavily on its oxidative metabolism to meet the demands placed by its energy-dependent osmotic and biosynthetic processes. Yet the kidney is only one of a number of organs that exhibit a near total reliance on oxidative metabolism for energy production and the question arises as to why, if direct inhibition of mitochondrial respiration is an important factor in the aetiology of phenacetin-related kidney damage, other aerobic tissues are not noticeably injured by the drug. Hence it is pertinent to ask whether kidney mitochondria are peculiarly susceptible to the inhibitory actions of phenacetin. It was to answer this question that the present investigation was undertaken.

The tissues chosen for study were kidney, liver and brain since it has been shown by other workers that mitochondria from these tissue differ in their sensitivities to the respiratory inhibitor tetrabutylammonium bromide (TBAB), a relatively lipophilic organic ion whose effects on respiration are similar, though not identical, to those of phenacetin [4]. It should be pointed out, however, that in the work with TBAB the brain mitochondrial preparation was obtained by a different method from that used for the isolation of mitochondria from the other tissues. Because the procedure used in the preparation of mitochondria might influence their respiratory activities and responses to inhibitors it was considered essential that, in order for legitimate comparisons to be made, mitochondria from all tissues should be obtained by the same method. Therefore mitochondria were isolated from cerebral cortex, kidney and liver of adult Wistar rats by the method of Clark and Nicklas [5] which, unlike conventional procedures, permits the isolation of brain mitochondria free from synaptosomal contamination. However, this method, which involves centrifugation through a discontinuous Ficoll–sucrose gradient, gave a much lower yield of mitochondria than do more orthodox procedures and so experiments were also performed with liver and kidney mitochondria isolated by the conventional method [6] used in our previous studies on phenacetin [3].

Respiratory rates were determined at 30° using a Rank oxygen electrode. Mitochondria (1.5–4 mg protein) were suspended in 2.5 ml of buffered medium [3] containing 4 mM L-glutamate, 4 mM L-malate and 1.6% ethanol, with or without 8 mM phenacetin. State 4 respiration was measured without further additions whereas State 3 (phosphorylating) respiration and State 3u (uncoupled) respiration were induced by the addition of 0.4 mM ADP and 0.04 mM 2,4-dinitrophenol respectively. Results are

Table 1. Effects of phenacetin on respiration in brain, kidney and liver mitochondria prepared by the method of Clark and Nicklas [5]

Tissue		nmol oxygen atoms consumed/min per mg protein State 4	State 3	State 3u	Respiratory control index
Brain	control	54 ± 2	119 ± 9	139 ± 11	2.20 ± 0.10
	+phenacetin	55 ± 2	83 ± 5*	104 ± 9*	1.51 ± 0.06†
	% inhibition by phenacetin	0	30 ± 3	25 ± 1	30 ± 3
Kidney	control	55 ± 4	119 ± 6	120 ± 11	2.20 ± 0.25
	+phenacetin	46 ± 1	56 ± 1†	65 ± 3†	1.22 ± 0.04*
	% inhibition by phenacetin	15 ± 5	52 ± 1	45 ± 3	44 ± 5
Liver	control	17 ± 3	90 ± 5	108 ± 4	5.56 ± 0.78
	+phenacetin	15 ± 2	30 ± 2†	51 ± 2†	2.04 ± 0.17†
	% inhibition by phenacetin	9 ± 4	66 ± 1	53 ± 2	63 ± 2

Mitochondria were incubated at 30° in 2.5 ml of the reaction medium containing 4 mM L-glutamate, 4 mM L-malate and 8 mM phenacetin where indicated. Respiration was determined with substrate only (State 4) and after addition of either 0.4 mM ADP (State 3) or 0.04 mM 2,4-dinitrophenol (State 3u). Results are given as mean values ± S.E.M. for between three and five separate experiments.

* Values with and without phenacetin are significantly different, $P < 0.05$.

† $P < 0.01$.

Table 2. Effects of phenacetin on respiration in kidney and liver mitochondria prepared by the method of Bustamante *et al.* [6]

Tissue		nmol oxygen atoms consumed/min per mg protein State 4	State 3	State 3u	Respiratory control index
Kidney	control	21 ± 1	156 ± 13	178 ± 18	7.68 ± 0.63
	+phenacetin	22 ± 1	65 ± 3†	99 ± 6*	2.89 ± 0.09†
	% inhibition by phenacetin	0	58 ± 2	44 ± 3	62 ± 4
Liver	control	10 ± 1	74 ± 5	92 ± 6	7.33 ± 0.83
	+phenacetin	8 ± 1	23 ± 2†	37 ± 3†	2.77 ± 0.29*
	% inhibition by phenacetin	18 ± 4	69 ± 2	60 ± 1	62 ± 3

Experimental conditions as for Table 1. Results are mean values ± S.E.M. for four separate experiments.

* Values with and without phenacetin are significantly different, $P < 0.01$.

† $P < 0.001$.

expressed as the mean ± S.E.M. and statistical significance was estimated by Student's *t*-test.

The data presented in Table 1 show the effects of phenacetin on respiration in mitochondria prepared by the Clark and Nicklas method. Phenacetin did not significantly alter State 4 respiration in any of the preparations but it inhibited to various degrees both State 3 and State 3u respiration in mitochondria from all three tissues. Inhibition of State 3 respiration in kidney mitochondria was significantly greater than in brain mitochondria ($P < 0.002$) but less than in liver mitochondria ($P < 0.002$). The inhibition of State 3u respiration, and also the depression of the Respiratory Control Index (RCI), used by some workers as the principal criterion in judging the efficacy of an inhibitor [4, 7, 8], followed the same pattern, brain mitochondria being the least affected and liver mitochondria the most.

When liver and kidney mitochondria were isolated by the conventional method of Bustamante *et al.* [6] the results shown in Table 2 were obtained. With liver mitochondria both State 4 and State 3 respiration were lower than in mitochondria prepared by the first method (cf. Table 1), while with kidney mitochondria there was a lower State 4 respiration but a higher State 3 respiration than in mitochondria prepared by the first method, giving rise to a much higher RCI. These results suggest that mitochondria prepared by the conventional method are better able to maintain their 'energized' condition and, at least in the case of kidney mitochondria, that the electron transport chain itself might be better preserved. Nevertheless, despite these differences in the respiratory capabilities of mitochondria prepared by the two procedures, their responses to phenacetin were essentially the same. Table 2 shows that, in mitochondria isolated by the second method, phenacetin did not affect State 4 respiration but strongly inhibited State 3 and State 3u respiration and markedly depressed the RCI. Except for the depression of the RCI, liver mitochondria were again significantly more susceptible than kidney mitochondria to phenacetin ($P < 0.01$).

The above results all show that although phenacetin exerts similar inhibitory effects on respiration in mitochondria from different tissues, not all mitochondria are equally sensitive to the drug. However, the order of sensitivity, viz. liver > kidney > brain, which is the same as that observed with TBAB and might be due to differences in the lipophilicity of the inhibitor binding site in the various mitochondria [4, 7], clearly indicates that kidney mitochondria are not especially susceptible to the inhibitory actions of phenacetin. It could be contended that, because renal cortex constituted the major bulk of the kidney tissue used in the preparation of mitochondria, there is the possibility that any special sensitivity to phenacetin of mitochondria from the renal medulla was masked. The medulla suffers most damage in analgesic nephropathy and studies have revealed that mitochondria from this region differ from renal cortical mitochondria in several respects, including the exhibition of a greater sensitivity to TBAB [8–11]. However, preliminary experiments in this laboratory have failed to reveal any significant differences between cortical and medullary mitochondria in their response to phenacetin. Hence, if direct inhibition of mitochondrial respiration is involved in the development of phenacetin-related renal disease, it is not due to a unique sensitivity of kidney mitochondria to the drug.

One possibility that remains is that kidney cells, particularly in the deeper regions of the organ, are normally exposed to higher concentrations of phenacetin than are the cells of other body tissues. This notion is supported by the data of Barraclough and Nilam [12] which indicated that phenacetin could be concentrated in the renal medulla *in vivo*, though Duggin and Mudge [13] suggested that it is not phenacetin which is concentrated but a labile conjugate of the drug. It appears, then, that more information is needed before an unequivocal answer can be given to the question of whether or not the direct effects of phenacetin on mitochondrial respiration are important in analgesic nephropathy.

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Release of proteases from cartilage cells as a result of activation by a macrophage factor—effects of some anti-inflammatory drugs

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An important facet of the pathology of rheumatoid arthritis is the degeneration of articular cartilage due to the active release of proteases by hypercellular synovial tissue and infiltrated mononuclear cells in the joint [1–5]. The extent of participation of chondrocytes in this process has not been determined. The clinical status of a typical osteoarthritic joint is different. The synovium exhibits little or no inflammation, only a few monocytes and lymphocytes have infiltrated into the joint, the levels of proteases in the synovial fluid are not significantly altered, and the degradation of cartilage-matrix occurs progressively over a long time period. Recent reports indicate that normal human articular cartilage contains low levels of metalloproteases [6]. The activities of cathepsin D, collagenase and other neutral proteases are high in osteoarthritic cartilage [7–9], although the mechanisms underlying these changes are not known. We have demonstrated recently that the chondrocytes from normal rabbit articular cartilage secrete very small amounts of proteases in their culture medium. Nevertheless, they can be activated repeatedly by a macrophage-derived inducer to produce high levels of these degradative enzymes [10]. The present study deals with the effects of various anti-inflammatory drugs on the induction of chondrocytic enzyme release using this *in vitro* model system.

The chondrocytes were isolated from articular cartilage of 2-month-old rabbits by enzymatic digestion, plated at a density of 3×10^5 cells/25 cm^2 flask, and grown to confluency in Ham's F-12 nutrient mixture, containing fetal calf serum (10% v/v) and antibiotics, in an atmosphere of 5% CO_2 in air [10].

The induction of cellular infiltration in the peritoneum of rabbits was carried out by an intraperitoneal injection of sterile light mineral oil. After 96 hr, the peritoneum was washed with Dulbecco's modified Eagle's medium (DMEM), containing 10% fetal calf serum (FCS), antibiotics, 2 mM glutamine and 2 units/ml of heparin. The lavage was centrifuged, the cells were washed twice with the same medium (without heparin), and then plated at a density of 1×10^6 cells/cm^2. After 4 hr, the medium and floating cells were removed. The adherent cells were mostly (at least 95 per cent) macrophages, as judged from their capability to phagocytoze oil droplets or latex particles. These cells were incubated overnight with 4 ml of DMEM (without serum), containing 30 μg/ml of lipopolysaccharide fraction (LPS) from *Escherichia coli* (Difco; 055:B5). The next day, the conditioned medium of macrophages (MCM) was removed and stored frozen [10].

The confluent chondrocytes were treated with MCM, diluted 1:5 with DMEM. Each chondrocyte-culture flask received 4 ml of the diluted medium. After a 48-hr incubation, the medium was dialyzed against 50 mM Tris-HCl buffer (pH 7.5), containing 200 mM CaCl, 5mM $CaCl_2$ and 0.02% NaN_3. Aliquots of the medium were treated with trypsin (100 μg/ml) for 10–15 min at 25° and then with soybean trypsin inhibitor (300 μg/ml) for 20 min at 25°, to convert any latent enzymes to their active forms. The collagenase activity in the medium was assessed by incubation with reconstituted fibrils of [^{14}C]glycine-labeled rat skin collagen (sp. act. 50,000–60,000 dis./min/mg) for 2 hr at 37°. The activities of other neutral proteases were estimated in the same manner, using [^{14}C]leucine-labeled globin as the substrate (sp. act. 15,000–20,000 dis./min./mg) [10].

The effects of various anti-inflammatory drugs on the release of these enzymes by chondrocytes were studied by adding them at various concentrations to the chondrocyte medium at the same time that MCM was added. Similar concentrations of the drugs were also added to the untreated chondrocytes as controls. After 48 hr at 37°, the media were dialyzed against Tris–HCl buffer to remove excess drugs. To assess the cytotoxic effects of the drugs, the total number of viable chondrocytes in each culture flask was estimated from their resistance for inclusion of trypan blue dye.

The results in Table 1 represent the inhibition of enzyme release by chondrocytes due to the presence of various drugs in the medium. As mentioned earlier, the confluent chondrocytes produced very small amounts of collagenase and other neutral proteases. The addition of MCM increased the enzyme secretion significantly during the 48-hr incubation period. To determine whether the chondrocytes synthesized a fraction of enzymes that was not released into the medium, the cells were isolated from the culture flasks with rubber policemen or by trypsin treatment. Trypsin was inactivated by the addition of excess soybean trypsin inhibitor or 10% FCS. The cells were frozen and thawed repeatedly, and the activities of collagenase and other neutral proteases in the cell-lysates were estimated after the treatment for conversion of any latent enzymes to the active forms. The levels of enzymes in the cell-lysates were undetectable, indicating that all the enzymes synthesized by chondrocytes were released into the medium.

Our previous findings had suggested that the optimum stimulatory effect on chondrocytes was observed when MCM was diluted 4- to 5-fold. Furthermore, the conditioned medium from the macrophages receiving no LPS showed minimal effect on the chondrocytes [10]. When LPS was added to the macrophages at various concentrations, 30 μg/ml of medium was found to the optimum concentration for maximum release of the stimulatory factor(s) (results not shown). The addition of varying amounts of LPS directly to the chondrocyte medium had no stimulatory or inhibitory effect on enzyme synthesis.

No significant effect was observed when the drugs were added to unstimulated chondrocytes. Paramethasone was extremely effective in suppressing the release of collagenase (approximately 70–80 percent inhibition) and, to a lesser extent, of neutral proteases by the MCM-treated chondrocytes. Aspirin was also very effective at 10^{-4} and 10^{-5} M