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EFFECTS OF INDOMETHACIN ON RESPIRATION AND THE α-GLYCEROLPHOSPHATE SHUTTLE IN RAT KIDNEY MITOCHONDRIA

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Abstract—The anti-inflammatory drug indomethacin was found to stimulate State 4 respiration in rat kidney mitochondria, indicating an uncoupler activity which was maximal at a concentration of 0.1–0.2mM. Indomethacin also inhibited State 3 respiration in mitochondria oxidizing glutamate or succinate, but not in mitochondria oxidizing ascorbate together with tetramethylphenylene diamine. This inhibition was not relieved by 2,4-dinitrophenol and suggested that indomethacin directly inhibited electron transport along the respiratory chain at a point prior to cytochrome *c*.

At concentrations one order of magnitude lower than that required for substantial uncoupling or respiratory inhibition, indomethacin severely restricted the transfer of reducing equivalents from extramitochondrial NADH to the respiratory chain via a reconstructed α-glycerolphosphate shuttle. It was found that the drug exerted a strong inhibitory effect on mitochondrial α-glycerolphosphate dehydrogenase activity, and that this inhibition was relatively specific since indomethacin had little effect on the activity of succinate dehydrogenase, another FAD-linked enzyme.

The inhibition of the α-glycerolphosphate shuttle is discussed in relation to the previously observed effects of indomethacin on glucose metabolism in isolated rat kidney tubules.

In an earlier communication [1] we reported that indomethacin brought about significant changes in the metabolism of glucose by isolated rat kidney tubules. The changes involved an increase in the rate of utilization of glucose and its conversion to lactate, accompanied by a marked fall in its conversion to CO_2. These effects were consistent with an indomethacin-induced rise in the cytoplasmic NADH/NAD^+ ratio but could not be accounted for satisfactorily by the previously observed actions of indomethacin on liver mitochondrial respiration [2–4]. It was therefore suggested that the drug might interfere with mechanisms responsible for the transfer of cytoplasmically-generated reducing equivalents to the mitochondrial respiratory chain.

In further investigations described here it was confirmed that indomethacin acts as both an uncoupler of oxidative phosphorylation and as an inhibitor of electron transport in kidney mitochondria as it does in liver mitochondria [2–4]. However, at concentrations at least one order of magnitude lower than that required for its maximum uncoupling effect, indomethacin severely restricted the ability of kidney mitochondria to oxidize extramitochondrial NADH via the α-glycerolphosphate shuttle [5]. This restriction was found to be due to a strong and specific inhibition of mitochondrial α-glycerolphosphate dehydrogenase by indomethacin.

MATERIALS AND METHODS

Chemicals. Indomethancin, NADH, ADP, rotenone, antimycin A, *N,N,N′,N′*-tetramethyl-*p*-phenylene diamine (TMPD), 2,6-dichlorophenol indophenol, DL-glycerol-3-phosphate, L-glycerol-3-phosphate and L-glycerol-3-phosphate:NAD^+ oxidoreductase (crystalline, Type X) were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.; L-ascorbic acid, sodium succinate and L-glutamic acid were from B.D.H. Ltd., Poole, Dorset, U.K.; 2,4-dinitrophenol was from Hopkin and Williams Ltd., Chadwell Heath, Essex, U.K.; phenazine methosulphate was from Calbiochem. San Diego, CA, U.S.A. All other reagents were A.R. grade.

Preparation of mitochondria and submitochondrial particles. Mitochondria were isolated from the kidneys of adult albino rats (Wistar strain) by a slight modification of the method of Bustamante *et al.* [6] as described elsewhere [7]. The medium used throughout the procedure comprised 220mM mannitol, 70mM sucrose, 2mM HEPES (*N*-2-hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid) and 0.05% (w/v) bovine serum albumin, pH 7.4. The mitochondrial preparation exhibited negligible lactate dehydrogenase activity [8] and very low glucose-6-phosphate hydrolase activity [9], indicating that it was essentially free from cytosolic and microsomal contamination.

Submitochondrial particles (SMP) were prepared by exposing the mitochondrial preparation to ultrasound for 2 min in a Branson B-12 Sonifier oscillating at 20 kHz with a power output of approx. 7 W. Unbroken mitochondria were removed by centrifugation at 25000 *g* for 10 min and the supernatant, which contained the SMP, was used without further treatment.

Oxygen consumption. Mitochondria (1–2 mg protein) were incubated at 30° in 2.5 ml of reaction mixture in the chamber of a Rank oxygen electrode (Rank Bros., Bottisham, Cambs., U.K.). The reaction medium was that detailed by Bustamante *et al.* [6] except for the omission of bovine serum albumin; substrates, ADP and dinitrophenol were added as indicated. Indomethacin was introduced as an ethanolic solution to give a final ethanol concentration of between 0.2 and 1 per cent (v/v).

Reconstruction of the α-glycerolphosphate shuttle. The shuttle was reconstructed using mitochondria (1–2 mg protein), L-glycerol-3-phosphate or DL-glycerol-

3-phosphate, rotenone, L-glycerol-3-phosphate:NAD^+ oxidoreductase and NADH as previously described [7]. The oxidation of extramitochondrial NADH was followed spectrophotometrically at 340 nm using a Varian Superscan 300 spectrophotometer. Indomethacin was added as indicated and the experiments were conducted at either 30° or 35°.

Enzyme assays. Succinate dehydrogenase and glycerol-3-phosphate dehydrogenase activities in mitochondria and SMP were assayed spectrophotometrically using a Varian Superscan 300 spectrophotometer operating in double beam mode. Both the sample and reference cuvettes contained, initially, intact mitochondria (0.2–1.5 mg protein) or SMP (0.2–1.0 mg protein) in 50mM potassium phosphate buffer (pH 7.4), 1mM KCN, 0.36mM phenazine methosulphate, 0.06mM 2,6-dichlorophenol indophenol (DCPIP) and up to 0.2mM indomethacin. The reaction was started by adding the substrate (2mM DL-glycerol-3-phosphate or 2mM succinate) to the reference cuvette, and the reduction of DCPIP was followed at 600 nm.

Protein. Protein was determined by a biuret method [10] with bovine serum albumin as the reference standard.

RESULTS

Effects of indomethacin on mitochondrial respiration. The effects of indomethacin on State 4 and State 3 respiration in mitochondria are shown in Fig. 1. Indomethacin stimulated State 4 respiration with either succinate (Fig. 1a) or glutamate (Fig. 1b) as substrate, the effect being maximal with 0.1–0.2mM indomethacin and indicating that the drug acted as an uncoupler of oxidative phosphorylation. In contrast, indomethacin inhibited State 3 respiration with both substrates. This inhibition could not be relieved by the addition of more ADP or by the uncoupler 2,4-dinitrophenol, indicating that indomethacin, as well as having uncoupler activity, inhibited electron transport by the respiratory chain.

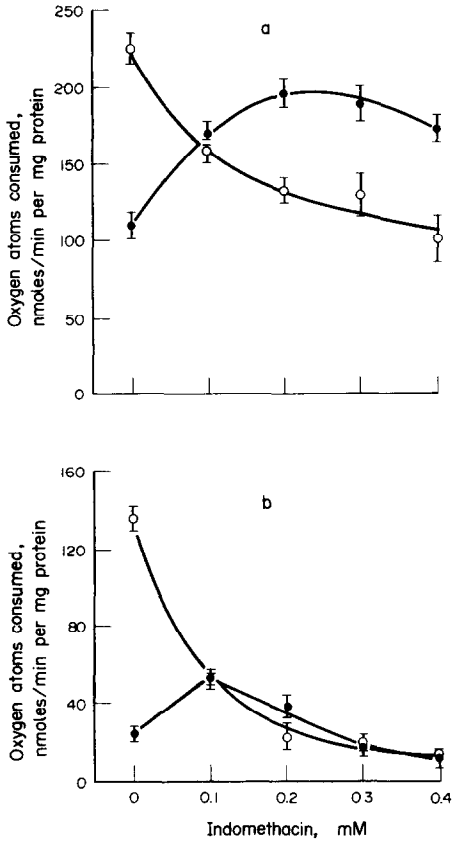

Fig. 1. Effect of indomethacin on respiration in kidney mitochondria. Mitochondria (1–2 mg protein) were incubated at 30° in 2.5 ml of reaction medium containing 8mM substrate, and up to 0.3mM indomethacin added as an ethanolic solution to give a final ethanol concentration of 0.2–1 per-cent (v/v). ADP was either omitted (●) or included at an initial concentration of 0.7mM (○). Results in Fig. 1a are with succinate, those in Fig. 1b are with glutamate. The points represent mean values ± S.E.M. for 3 separate experiments.

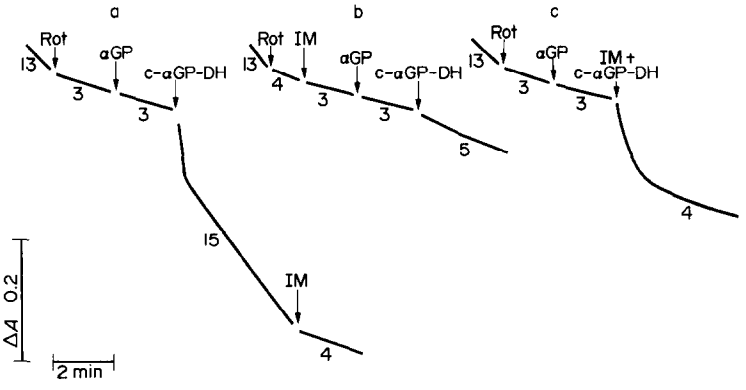

Fig. 2. Effect of indomethacin on the oxidation of added NADH by kidney mitochondria. Mitochondria (1.7 mg protein) were incubated at 35° in 2.5 ml of reaction medium containing 0.12mM NADH. The additions were 5μM rotenone (Rot), 0.4mM L-glycerol-3-phosphate (αGP), 2 units of L-glycerol-3-phosphate:NAD^+ oxidoreductase (*c*-αGP-DH) and 0.1mM indomethacin (IM). The trace shows the decrease in absorbance at 340 nm and the figures alongside give the rate of NADH oxidation in nmoles/min per mg mitochondrial protein.

This inhibitory effect on State 3 respiration was not observed when the substrate was ascorbate together with TMPD (data not shown), suggesting that the inhibition of electron transport occurred at a point prior to cytochrome *c*.

Effect of indomethacin on the oxidation of extramitochondrial NADH via the α-glycerolphosphate shuttle. In order to determine whether indomethacin might alter the ability of mitochondria to accept reducing equivalents from added NADH, and whether any such alteration could be attributed to the actions of the drug on respiration, experiments with the reconstructed α-glycerolphosphate shuttle were undertaken. The results of three different experiments performed at 35° are presented in Fig. 2 and they demonstrate that indomethacin exerted a strong inhibitory effect on the mitochondrial section of the shuttle. Figure 2a shows that the rate of rotenone-insensitive NADH oxidation increased when L-glycerol-3-phosphate:NAD^+ oxidoreductase (*c*-αGP-DH) was added to a system containing mitochondria, L-glycerol-3-phosphate (L-αGP) and NADH. The initial sharp decrease in absorbance was due to the immediate *c*-αGP-DH-catalysed oxidation of NADH by dihydroxyacetone phosphate (DHAP) which had accumulated in the medium owing to the oxidation of L-αGP by the mitochondria. The subsequent sustained rate of NADH oxidation, which reflected the continuing production of DHAP and thus indicated precisely the rate of operation of the α-glycerol-phosphate shuttle, was strongly inhibited when 0.1mM indomethacin was added. In the experiment shown in Fig. 2b the introduction of indomethacin prior to the addition of L-αGP caused the virtual elimination of both the rapid phase and the sustained phase of NADH oxidation. This indicated that indomethacin interfered with the overall operation of the shuttle but did not reveal whether the inhibition was exerted on the cytoplasmic arm (DHAP reduction) or the mitochondrial arm (L-αGP oxidation) of the shuttle. However, if indomethacin was added after L-αGP and just prior to the introduction of *c*-αGP-DH, as in the experiment in Fig. 2c, the rapid phase of NADH oxidation occurred and only the later sustained rate was abolished. This showed that the added *c*-αGP-DH activity was not affected by indomethacin and that the production of DHAP, which occurred at a normal rate before indomethacin was added, was strongly inhibited by the drug, clearly indicating an inhibition of L-αGP oxidation by the mitochondria.

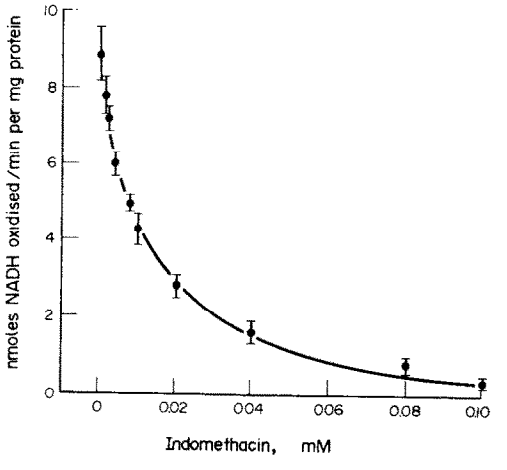

Fig. 3. Effect of indomethacin on the oxidation of added NADH by the reconstructed α-glycerolphosphate shuttle. Conditions were as described for Fig. 2 except that 0.4mM DL-glycerol-3-phosphate was used and the experiments were performed at 30°. Additions were made in the order shown in Fig. 2a and the results represent the mean values ± S.E.M. for 3 separate experiments.

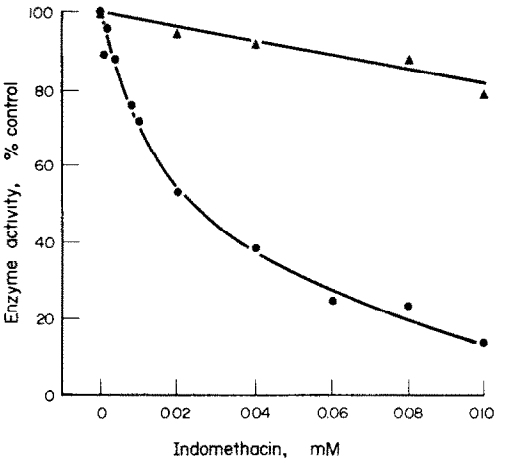

Fig. 4. Effects of indomethacin on mitochondrial L-glycerol-3-phosphate dehydrogenase and succinate dehydrogenase activities. Intact mitochondria or submitochondrial particles were incubated at 30° in 2.5 ml of 50mM phosphate buffer (pH 7.4) containing 1mM KCN, 0.36mM PMS and 0.06mM DCPIP as described under Methods. Activities are expressed as percentages of the activity measured in the absence of indomethacin. The lines indicate L-glycerol-3-phosphate dehydrogenase in intact mitochondria (●) and succinate dehydrogenase in submitochondrial particles (▲).

In Figure 3 are the results of experiments conducted at 30° with DL-αGP and various concentrations of indomethacin. It can be seen that 50 per cent inhibition of the α-glycerolphosphate shuttle activity was brought about by 0.01mM indomethacin. This may be compared with the 0.1–0.2mM indomethacin required for full uncoupling activity and the 0.1–0.3mM indomethacin needed for 50 per cent inhibition of State 3 respiration (see Fig. 1). The greater sensitivity of the shuttle to inhibition by indomethacin suggested that it could not be accounted for adequately by either the uncoupler action or the electron transport inhibitor action of the drug.

Effects of indomethacin on mitochondrial glycerol-3-phosphate dehydrogenase and succinate dehydrogenase activities. The flavin-linked L-glycerol-3-phosphate dehydrogenase (*m*-αGP-DH) is located at the outer surface of the mitochondrial inner membrane and is free to interact with electron acceptors, such as phenazine methosulphate (PMS), added to the external medium. If the transport of electrons from L-αGP to oxygen is blocked by cyanide, their rate of transfer to the reducible dye DCPIP via PMS gives a measure of *m*-αGP-DH activity. Figure 4 shows that indomethacin strongly inhibited the reduction of DCPIP by mitochondria oxidizing DL-αGP in the presence of cyanide. Similar results were obtained when SMP were used instead of intact mitochondria (data not shown). Approximately 0.02mM indomethacin gave 50 per cent

inhibition of DCPIP reduction, a concentration similar to that required for 50 per cent inhibition of the α-glycerolphosphate shuttle (see Fig. 3).

In order to determine whether this effect was specific for *m*-αGP-DH or occurred also with other FAD-linked dehydrogenases the activity of succinate dehydrogenase was also assayed. Preliminary experiments revealed that in intact mitochondria this enzyme, which is located at the inner surface of the mitochondrial inner membrane, was not fully accessible to PMS and that some of the electrons transferred from succinate to DCPIP via PMS were derived from reduced cytochrome *c*. This conclusion was based on the observations (not shown) that antimycin A inhibited DCPIP reduction by succinate and that indomethacin, at concentrations up to 0.1mM, slightly stimulated the rate of DCPIP reduction, consistent with the uncoupling effect described earlier. However, when SMP, in which succinate dehydrogenase is easily accessible to PMS, were used for the assay, indomethacin had only a very slight inhibitory effect on the rate of reduction of DCPIP (Fig. 4). These results showed that the inhibitory effects of low concentrations of indomethacin were relatively specific for *m*-αGP-DH.

DISCUSSION

The α-glycerolphosphate shuttle, because of its unique arrangement [11], is the simplest and most direct means for the transfer of cytoplasmically-generated reducing equivalents to the mitochondrial respiratory chain. Its importance in aerobic glucose metabolism has been demonstrated in insect flight muscle [12] and certain types of tumour cell [13], and has been inferred for a number of mammalian tissues [14–17]. In an earlier paper describing the reconstruction of the α-glycerophosphate shuttle with rat kidney mitochondria we pointed out that it might also play a significant role in renal glucose metabolism [7]. If true, it can be predicted that any interference with its operation would lead to changes in the metabolism of glucose in kidney preparations.

The anti-inflammatory drug indomethacin was previously shown to have a marked influence on glucose metabolism in isolated rat kidney tubules, causing glycolytically-produced pyruvate to be diverted away from CO_2 production and towards lactate production [1]. This diversion was not easily explicable in terms of the reported actions of indomethacin on respiration in liver mitochondria [2–4], and instead appeared to imply a rise in the cytoplasmic NADH/NAD^+ ratio. Since there was no evidence of general respiratory inhibition it was suggested that a rise in this ratio could stem from an interference with the transfer of reducing equivalents, produced by the glyceraldehyde-3-phosphate dehydrogenase reaction, to the respiratory chain. The results now reported appear to support this contention in that indomethacin restricted the oxidation of extramitochondrial NADH via the reconstructed α-glycerolphosphate shuttle. Although it was the mitochondrial arm of the shuttle that was affected the inhibition could not be ascribed to either an uncoupling of oxidative phosphorylation or an inhibition of the respiratory chain, both of which actions were significant in kidney mitochondria only at indomethacin concentrations 10 times higher than that required for inhibition of the shuttle. Investigation of mitochondrial α-glycerolphosphate dehydrogenase activity revealed that it was this enzyme, previously identified as the rate-limiting step of the reconstructed shuttle [7], which was inhibited by indomethacin. The inhibition was specific to the extent that succinate dehydrogenase, another FAD-enzyme, was almost unaffected by indomethacin.

It therefore appears that indomethacin, through its inhibition of mitochondrial glycerolphosphate oxidation, and hence of the α-glycerolphosphate shuttle, could cause an increase in the cytoplasmic NADH/NAD^+ ratio in the intact kidney cell. This effect would be independent of the other actions of indomethacin on mitochondrial respiration and could explain why glucose is metabolized primarily to lactate rather than CO_2 when the drug is present. It should be noted, however, that kidney cells contain the enzymes necessary for the transfer of reducing equivalents via the malate–aspartate shuttle [18] and the fatty acid shuttle [19], though the significance of these shuttles relative to the α-glycerolphosphate shuttle, and the degree to which they could compensate for a loss of α-glycerolphosphate shuttle activity, remain to be investigated. Indeed, examination of all the shuttles, their interactions, and the effects of indomethacin on them may be necessary in order to gain a more complete understanding of the way in which indomethacin influences glucose metabolism in the kidney cell.

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