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# Action of diclofenac on kidney mitochondria and cells

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**Abstract**

The mitochondrial membrane potential measured in isolated rat kidney mitochondria and in digitonin-permeabilized MDCK type II cells pre-energized with succinate, glutamate, and/or malate was reduced by micromolar diclofenac dose-dependently. However, ATP biosynthesis from glutamate/malate was significantly more compromised compared to that from succinate. Inhibition of the malate–aspartate shuttle by diclofenac with a resultant decrease in the ability of mitochondria to generate NAD(P)H was demonstrated. Diclofenac however had no effect on the activities of NADH dehydrogenase, glutamate dehydrogenase, and malate dehydrogenase. In conclusion, decreased NAD(P)H production due to an inhibition of the entry of malate and glutamate via the malate–aspartate shuttle explained the more pronounced decreased rate of ATP biosynthesis from glutamate and malate by diclofenac. This drug, therefore affects the bioavailability of two major respiratory complex I substrates which would normally contribute substantially to supplying the reducing equivalents for mitochondrial electron transport for generation of ATP in the renal cell.


*Keywords:* Diclofenac; Malate–aspartate shuttle; ATP biosynthesis; Mitochondrial membrane potential; NAD(P)H; NSAIDs

Diclofenac (2-[(2,6-dichlorophenyl)amino]phenylacetate) is one of the most frequently used non-steroidal anti-inflammatory drugs (NSAIDs) prescribed to millions of people worldwide for the treatment of osteoarthritis, rheumatoid arthritis, and muscle pain. Although its toxicity is commonly associated with the gastrointestinal tract [1] and with idiosyncratic hepatic injury [2], there appeared to be a direct link of diclofenac to severe, acute renal failure in vultures in the Indian subcontinent [3–5]. The dramatic loss of >95% of the vulture population from 1992 coincided with the introduction of this NSAID in the feed of livestock over the same period. Vultures in the laboratory fed diclofenac exhibited similar acute renal failure. It was concluded that the massive scale of diclofenac poisoning was due to the fact that this drug was concentrated in the kidney and liver of domestic livestock, and vultures fed on these organs of the carcasses. Post-mortems on the affected vultures showed kidney failure with accumulation of uric acid in the visceral organs [3]. To-date the mechanism of killing of vultures remains unknown although the kidney appears to be the main target. A literature search on diclofenac-induced toxicity associated with acute renal failure in humans retrieved a number of isolated cases [6–14]. Diclofenac also damages the kidneys of rainbow trout and rabbits [15–17]. Ingested diclofenac residues were found to be more concentrated in the kidneys of buffalo and goat compared to their respective livers by a factor of 4, and its bio-accumulation in the kidneys of the affected vultures was also noted [3]. Interestingly, studies of diclofenac-induced nephrotoxicity *in vivo* showed oxidative stress and genomic DNA fragmentation, but there was no liver damage [18]. Whatever the mechanism/s, diclofenac seems to have a more adverse effect on the kidneys in various animals including human.

## Materials and methods

*Chemicals*. The following chemicals were from Sigma Aldrich Co Ltd, USA: ATP, ADP, amino-oxyacetate (AOA), L-aspartate, decylubiquinone, diclofenac sodium, L-glutamic acid, KCN, L-malic acid, malate

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dehydrogenase (porcine heart, 1450 U/mg), $NAD^+$ and its reduced form NADH, oligomycin, rotenone, succinate, and FL-ASC Bioluminescent cell assay kit. Aspartate transaminase (porcine heart, 1276 U/ml) was from Fluka Chemie (Buchs, Switzerland). JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolocarbocyanine iodide) was from Molecular Probes Inc. (Eugene, OR, USA).

*Cell culture and sample preparation*. The Madin–Darby canine kidney (MDCK type II) cells were a gift from Dr. Walter Hunziker of Institute of Molecular and Cell Biology, Biopolis, Singapore. These cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 100 U/ml each of penicillin G and streptomycin and 0.25 μg amphotericin B supplemented with 10% fetal bovine serum (FBS) in a humidified incubator with 5% $CO_2$ at 37 °C. Following trypsinization, the cells were resuspended in Medium A which contained 250 mM sucrose, 20 mM Hepes, and 10 mM $MgCl_2$ at pH 7.1. Prior titration with digitonin for permeabilization of the MDCK cells to facilitate the entry of respiratory substrates was carried out and an optimal concentration of 3 μg digitonin/million cells was established. The action of digitonin was stopped by the addition of an equal volume of 0.3% bovine serum albumin [19].

*Isolation of mitochondria from rat kidneys*. Our method of isolating mitochondria from rat kidneys has been reported previously [20]. Protein determination was carried out by the Bradford procedure [21].

*ATP measurement in isolated mitochondria*. This was based on the luciferin-luciferase reaction following our earlier reports [20,22]. The initial rate of ATP biosynthesis was measured at 5 min and the chemiluminescence was determined in a luminometer (Perkin-Elmer, Victor 3). Respiratory substrates with their final concentrations given in parentheses were employed in the absence or presence of 5–50 μM diclofenac: succinate (10 mM) plus 5 μM rotenone, glutamate/malate (each at 5 mM added singly or together).

*Mitochondrial membrane potential*. JC-1, a mitochondrial specific probe, was used as reported previously [20,22]. The mitochondrial membrane potential (MMP) was monitored following the uptake of JC-1 into isolated kidney mitochondria, using a Perkin-Elmer LS55 luminescence spectrometer (Buckinghamshire, UK). The simultaneous scanning of the green monomers and red J-aggregates by the dual-wavelength mode has been described in detail in our earlier paper for measurement of MMP in isolated brain mitochondria [22]. The procedure has been extended in this study to MDCK cells to provide a whole cell approach. The cells were first permeabilized by digitonin to facilitate the entry of the respiratory substrates. The MMP was monitored in 2 ml of a respiratory buffer (pH 7.1) containing 20 mM Hepes, 250 mM sucrose, 10 mM $MgCl_2$, and 12.5 mM $KH_2PO_4$, and 0.2 μM JC-1 was then added. Energization was initiated with 10 mM succinate or 5 mM each of glutamate/malate. On establishing an optimal J-aggregate peak, 5–100 μM diclofenac was introduced and changes in the MMP monitored for 4–6 min were compared to the corresponding controls without diclofenac.

*NADH dehydrogenase activity*. The rotenone-sensitive reduction of decylubiquinone was measured following the procedure reported for respiratory complex I activity [23] using a freeze-thawed mitochondrial extract (containing 0.3 mg protein) obtained from the isolated intact mitochondria prepared by differential centrifugation as described [20]. The decrease in the auto-fluorescence of NADH was monitored fluorimetrically at Ex/Em of 352/464 nm in the absence or presence of 100 μM diclofenac or 5 μM rotenone which served as a positive control.

*GDH and MDH activities*. Both activities were measured by the changes in fluorescence of NADH at Ex/Em of 352/464 nm in a microplate reader (Spectramax, Gemini XS, Molecular Devices) using extracts of isolated intact mitochondria containing 0.2 and 0.02 mg protein (preincubated in the absence or presence of 10–100 μM diclofenac), respectively, for the GDH and MDH assays [24–26]. The concentrations of the substrates for the GDH assay are 10 mM α-ketoglutarate and 0.2 mM NADH for the forward reaction and 20 mM glutamate with 5 mM $NAD^+$ for the reverse reaction. The MDH assays were carried out with 0.16 mM NADH and 0.4 mM oxaloacetate in the forward, and 1 mM $NAD^+$ and 5 mM malate in the reverse directions. Initial velocities were recorded for the first 5 min at room temperature of 24 °C.

*Measurement of the malate–aspartate shuttle*. The shuttle capacity was measured in isolated rat kidney mitochondria by reconstituting the extramitochondrial compartment as described [27]. Essentially the assay mixture contained aspartate (2 mM), aspartate transaminase (2 U/ml), ADP (2 mM), MDH (3 U/ml), and 0.035 mM NADH. Baseline NADH oxidation was measured fluorimetrically at Ex/Em of 352/464 nm for 4 min upon addition of intact kidney mitochondria containing 0.3 mg protein. The malate–aspartate shuttle activity was initiated by adding 5 mM glutamate/malate. The difference in activity before and after the addition of glutamate/malate was taken as the shuttle capacity. Aminooxyacetate, an inhibitor of transaminase [28], was included as a positive control. To examine the type of inhibition, kinetic studies were carried out with 0.5–5 mM each of glutamate/malate in the absence or presence of 5 and 50 μM diclofenac.

*Measurement of intra-mitochondrial NAD(P)H generated from glutamate/malate*. The basal NAD(P)H was measured in a Perkin-Elmer LS55 luminescence spectrometer at Ex/Em of 352 nm/464 nm upon the addition of a preparation of intact kidney mitochondria (containing 0.3 mg protein) pre-incubated with 2 μg oligomycin/ml to inhibit $F_1F_o$ ATPase; with this inhibition, respiration in tightly coupled mitochondria was also inhibited [29]. The RCR (respiratory control ratio) routinely measured in our kidney mitochondrial preparations with glutamate/malate ranged from 5.5 to 7. The change in auto-fluorescence of NAD(P)H attributed to the uptake of glutamate/malate (5 mM each) was monitored and compared to those in aliquots of mitochondrial preparation which had been pre-incubated with 5–50 μM diclofenac for 5 min. The amount of NAD(P)H produced was extrapolated from a standard curve containing 2–10 nmol NADH which showed a linear response. The stock solutions of glutamate and malate were prepared in Tris–HCl and adjusted to pH 7.1 to minimize their background fluorescence.

*Statistical analysis*. Data are presented as means ± SD of triplicates. $p$ values smaller than 0.05 were considered to be statistically significant.

## Results

### *ATP biosynthesis in isolated rat kidney mitochondria*

The effects of diclofenac on the rate of ATP biosynthesis in isolated rat kidney mitochondria measured at 5 min are shown in Fig. 1. Inhibition of ATP biosynthesis from the oxidation of glutamate and/or malate by diclofenac was concentration-dependent between 5 and 50 μM, and inhibition was evident at 5 and 10 μM diclofenac. However with succinate as the oxidizable substrate, at least 50 μM diclofenac was required to produce a significant decrease which was much smaller in magnitude when compared to glutamate and/or malate (Fig. 1).

### *Loss of mitochondrial membrane potential*

#### *In isolated kidney mitochondria*

Uptake of JC-1 into mitochondria isolated from freshly excised kidneys was demonstrated by an increase in the green fluorescence of the monomers (shown by dotted lines in Fig. 2a) with concomitant formation of the J-aggregates represented by an increase in the red fluorescence. Upon addition of 10 mM succinate, an immediate sharp increase in MMP was reflected by the higher intensity of the red fluorescence signal with a reciprocal decrease in the green fluorescence (shown in dotted lines for control and a representative diclofenac concentration of 25 μM in Fig. 2a). The established MMP was maintained for about 5–6 min.