Effects of indomethacin on energy metabolism in rat and human jejunal tissue *in vitro*

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**ABSTRACT**
Non-steroidal anti-inflammatory drugs (NSAIDs) are known to cause enteropathy, but the mechanism by which this toxicity occurs is less well established. This paper sets out to test the hypothesis that these drugs affect oxidative phosphorylation in jejunal tissue, thereby interfering with energy metabolism and rendering the tissue vulnerable to damage. Jejunal tissue obtained from rats and humans was used for *in vitro* determinations of oxygen uptake, lactate production and energy charge levels in the presence of indomethacin, a commonly used NSAID. In the rat jejunal tissue, drug concentrations of 0.5 mM and 2.5 mM produced significant decreases in oxygen uptake (*P* < 0.01) and energy charge levels in the tissue (*P* < 0.05). There was a corresponding increase in lactate production by the tissue at these indomethacin concentrations (*P* < 0.05). Rat jejunum examined by electron microscopy after incubation with various concentrations of indomethacin showed ultrastructural effects of the drug on mitochondrial morphology. In human tissue, an inhibitory effect of indomethacin on oxygen uptake was seen, but the effects on lactate production and energy charge were less conclusive. These findings suggest that indomethacin affects mitochondria and thereby impairs energy metabolism in jejunal tissue.

**INTRODUCTION**
Non-steroidal anti-inflammatory drugs (NSAIDs) are among the most widely prescribed groups of drugs world-wide. However, their side effects are a cause for concern, especially those that affect the gastrointestinal tract. It has been suggested that up to 60–70% of patients on long-term NSAIDs may have asymptomatic enteropathy [1]. The intestinal inflammation that constitutes NSAID-induced enteropathy is associated with complications that include intestinal bleeding, intestinal protein loss [2] and small-intestinal strictures [3,4].

The precise mechanisms by which NSAIDs cause enteropathy are not well established. Inhibition of cyclo-oxygenase, resulting in low levels of prostaglandins, is an important component, but the pathogenesis is also believed to include a non-prostaglandin-dependent ‘topical effect’. The importance of the latter has recently been re-addressed. It is clear that several NSAIDs, such as aspirin and indomethacin, uncouple mitochondrial oxidative phosphorylation [5]. Studies with these and other acidic NSAIDs (but not with some of the selective cyclo-oxygenase-2 inhibitors, presumably because they are non-acidic [6]) in isolated mitochondrial preparations

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**Key words:** ATP, energy charge, mitochondria, non-steroidal anti-inflammatory drugs, NSAIDs, oxidative phosphorylation.  
**Abbreviations:** NSAID, non-steroidal anti-inflammatory drug; DNP, dinitrophenol.  
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have shown that they uncouple oxidative phosphorylation at low concentrations and inhibit respiration at higher concentrations [6–8], thereby affecting energy metabolism. It has been suggested that this, in turn, leads to disruption of the intercellular barrier function, with a resultant increase in intestinal permeability, thereby leading to inflammation. The mitochondrial effects of NSAIDs are distinctive from their inhibitory effect on cyclo-oxygenase. The former effect leads to increased small-intestinal permeability and inflammation, whereas inhibition of gastric [9,10] or intestinal [11,12] cyclo-oxygenase by parenteral aspirin is not associated with disruption of the intestinal barrier function or with inflammation. However, at present there is little evidence that NSAIDs, through either uncoupling of oxidative phosphorylation or inhibition of the respiratory chain, lead to decreased intestinal ATP levels. One problem with obtaining such information is the difficulty in obtaining suitably coupled preparations of these organelles from gut tissue [13]. We therefore set out to investigate the hypothesis that NSAIDs uncouple or inhibit oxidative phosphorylation, using intact jejunal tissue in vitro.

**MATERIALS AND METHODS**

**Animals and tissues**

In order to allow comparisons with previously published work and with human tissues, we examined rat jejunal tissue obtained from living rats under surgical anaesthesia (i.e. similar conditions to tissue obtained from humans), and also rat jejunum from rats freshly killed by cervical dislocation (i.e. similar conditions to tissue obtained from humans), and also rat jejunum from rats freshly killed by cervical dislocation (i.e. similar to tissue used for the preparation of isolated mitochondria).

Male Sprague–Dawley rats (body weight 200–250 g) were obtained from Charles River UK Ltd, and were used as the source of rat jejunal tissue. All procedures on animals were undertaken according to the requirements laid down by the U.K. Home Office. Indomethacin (Sigma) was the NSAID studied. 2,4-Dinitrophenol (DNP) (BDH), a classical uncoupler, was used for purposes of comparison. Both drugs were suspended in 10% (v/v) DMSO. Indomethacin at 0.1 mM, 0.5 mM and 2.5 mM, and DNP at 0.1 mM. Both drugs were suspended in 10% (v/v) DMSO.

Mitochondria-specific oxygen uptake rates were calculated in each case by subtracting the rate of uptake in the presence of cyanide from both the baseline rate and the rate in the presence of the drug.

**Determination of oxygen uptake by jejunal tissue**

Each jejunal fragment was incubated immediately in 2 ml of oxygenated buffer in the sample chamber of a Clarke oxygen electrode. For the incubation, a Hepes buffer (pH 7.4) of the following composition was used: 125 mM NaCl, 3.5 mM KCl, 16 mM Hepes, 10 mM MgCl₂, 1 mM CaCl₂ and 10 mM glucose as respiratory substrate. The rates of oxygen uptake, both at baseline and in the presence of the drug, were each measured over 5 min periods; rates were also measured in the presence of cyanide to determine mitochondria-specific uptake. The final concentrations of drugs used were as follows: indomethacin at 0.1 mM, 0.5 mM and 2.5 mM, and DNP at 0.1 mM. Both drugs were suspended in 10% (v/v) DMSO.

Mitochondria-specific oxygen uptake rates were calculated in each case by subtracting the rate of uptake in the presence of cyanide from both the baseline rate and the rate in the presence of the drug.

**Determination of lactate production by jejunal tissue**

The incubation buffer used for the oxygen uptake measurements was sampled to estimate lactate production by the tissue before and after the addition of each concentration of the drug. The assay for lactate was carried out by the spectrophotometric method of Beutler [14] with lactate dehydrogenase.

**Determination of energy charge levels in jejunal tissue**

Jejunal tissue that had been incubated for 5 min in buffer with each of the concentrations of the drugs was freeze-clamped with tongs that had been pre-cooled in liquid nitrogen. The frozen specimens of jejunum were ground up under liquid nitrogen, using a pre-cooled mortar and pestle. They were thawed in 10% (v/v) perchloric acid. The samples were centrifuged at 10000 g for 10 min. The resultant supernatant was collected and neutralized using 3 M K₂CO₃ with Methyl Orange as indicator. A blank perchloric acid extract without tissue was prepared with each batch. The three adenine nucleotides (AMP, ADP and ATP) were assayed using the spectrophotometric methods of Beutler [14]. Internal standards were run for each nucleotide and corrections were made for any
systematic losses. The energy charge of the sample was calculated as follows [15]:

\[
\text{Energy charge} = \frac{0.5[\text{ADP}] + [\text{ATP}]}{[\text{ATP}] + [\text{ADP}] + [\text{AMP}]}
\]

**Determination of oxygen uptake, lactate production and energy charge levels in human jejunal tissue**

Normal human jejunal tissue was obtained from biopsies done on patients posted for endoscopy in the Day Surgery Centre of King’s College Hospital, London. Informed consent was obtained from the patients. Permission for use of human tissue for experimentation had been obtained from King’s Healthcare Ethical Committee under the licence of I.B.

The fragments of tissue obtained were used in experiments as described above to determine oxygen uptake, lactate production and energy charge levels in the tissue.

**Determination of mitochondrial morphology**

Rat jejunal tissue was prepared for transmission electron microscopy as follows. Fragments of tissue, which had been incubated in the Hepes buffer, pH 7.4, at 37 °C for 10 min, were put into Karnovsky fixative at 4 °C for 1–2 h to achieve fixation. It was then placed into cacodylate/sucrose solution overnight. Post-fixation was carried out in 1% osmium tetroxide for 1 h at 4 °C. After a brief rinse in distilled water, the tissue was dehydrated through a graded series of ethanol (10%, 50%, 70% and absolute alcohol) and eventually epoxypropane. The tissue was then embedded in araldite. Ultrathin sections were cut using an Ultratome Richart Ultracut-E. They were then examined with a Joel 1200 cm electron microscope in transmission mode.

**RESULTS**

Figure 1 shows that DNP (0.1 mM) stimulated respiration significantly in rat and human jejunum. Indomethacin significantly decreased respiration at concentrations of 0.5 and 2.5 mM in tissue from anaesthetized rats, and at 2.5 mM in tissue from killed rats and from humans. There were no significant differences between rat jejunum taken directly from live animals under general anaesthesia and tissue taken from rats killed shortly before (< 2 min) tissue harvesting.

Indomethacin had no significant effect on lactate production in rat or human jejunum at concentrations of 0.1–0.5 mM, except that there was a significant increase in tissue from anaesthetized rats at 0.5 mM indomethacin (Figure 2). Both indomethacin at 2.5 mM and DNP at 0.1 mM increased lactate production significantly in both the rat preparations, but not in human tissue. Thus no effect of indomethacin on lactate production was seen in human jejunum.

Figure 3 shows that energy charges were higher in tissue obtained from anaesthetized rats compared with that from killed ones. In tissue from anaesthetized rats, the energy charge was significantly decreased after incubation with indomethacin (0.5–2.5 mM) or DNP, while in tissue from killed rats only the highest concentration of indomethacin and DNP decreased the energy charge significantly. The energy charge was not significantly affected by indomethacin or DNP in human jejunal tissue.
Figure 3  Effects of indomethacin and DNP on energy charge of jejunal tissue in vitro
Data are means ± S.E.M. (n = 6); *P < 0.05, **P < 0.01 compared with control (Student’s t-test).

Table 1  Effects of indomethacin and DNP on morphology of mitochondria from rat jejunum in vitro

Random electron microscopic pictures were taken of tissue that had been incubated with each of the concentrations of indomethacin, DNP or control solution (10% DMSO). All mitochondria in each of these pictures were counted, and the percentage of damaged mitochondria was calculated. Data in the final column are means (S.E.M.): **P < 0.01, ***P < 0.001 compared with controls (Student’s t-test).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Total number of mitochondria counted</th>
<th>Damaged mitochondria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>428</td>
<td>362 (84)</td>
</tr>
<tr>
<td>Indomethacin (0.1 mM)</td>
<td>379</td>
<td>372 (97)</td>
</tr>
<tr>
<td>Indomethacin (0.5 mM)</td>
<td>402</td>
<td>371 (92)</td>
</tr>
<tr>
<td>Indomethacin (2.5 mM)</td>
<td>440</td>
<td>429 (98)</td>
</tr>
<tr>
<td>DNP (0.1 mM)</td>
<td>523</td>
<td>460 (89)</td>
</tr>
</tbody>
</table>

After a 10 min incubation in Hepes buffer, a high proportion of mitochondria were abnormal in control tissue (84%), as shown in Table 1. Nevertheless, all concentrations of indomethacin were associated with significantly increased ultrastructural mitochondrial damage, while DNP-treated tissue did not differ significantly from control tissue.

DISCUSSION

The data show that effects of the classical uncoupler, DNP, on energy metabolism in rat jejunal tissue can be detected. Significant effects were apparent on oxygen consumption rates, lactate production and energy charge. Similar effects were seen with human tissue. However, in the latter case, due to inter-individual variation, these effects were statistically significant only for oxygen consumption data. When indomethacin was used instead of DNP, there was no significant stimulation of oxygen uptake in rat or human jejunal tissue. Inhibition of oxygen uptake was, however, seen at 2.5 mM indomethacin in jejunum from both species, and also at 0.5 mM indomethacin in tissue from anaesthetized rats.

The energy content of the tissue was measured in terms of the energy charge of the adenylate pool, a parameter that is intended to furnish a quantitative estimate of the energy state of the cell [15]. Significant falls in energy charge were seen only in rat tissue, and both indomethacin and DNP were effective in this respect (Figure 3).

Several NSAIDs, such as aspirin and indomethacin, are known to be uncouplers [5]. Studies with these two drugs and a range of other acidic NSAIDs in isolated mitochondrial preparations have shown that they uncouple oxidative phosphorylation at concentrations between 0.03 mM and 1.5 mM (depending on the particular NSAID) and inhibit respiration at higher concentrations [6–8]. However, similar concentrations of the drugs showed no significant uncoupling effect in the present study. Indeed, the only effect seen was an inhibitory effect at high concentrations.

In order to investigate this finding further, the morphology of mitochondria in these jejunal fragments was studied. From Table 1, it can be seen that the control group had a very high proportion of damaged mitochondria. Thus it is possible that the mitochondria in the enterocytes were already uncoupled after this relatively short incubation in vitro. This may account for the small rises in oxygen uptake produced by DNP and absence of an effect of indomethacin. It is, however, also clear from Table 1 that it was still possible to detect an ultrastructural effect of indomethacin at all concentrations of the drug in vitro. This is in accordance with in vivo studies that have shown ultrastructural damage to intestinal mitochondria from rats after the animals had been dosed orally with indomethacin [8] and other acidic NSAIDs such as flurbiprofen [16].

Significant elevations in lactate levels in the jejunal tissue were seen in response to indomethacin at 0.5 mM and 2.5 mM. The rationale for assessing lactate production is that the inhibition of oxidative phosphorylation that occurs at these concentrations should lead to low ATP/ADP and high NADH/NAD+ ratios in the tissue. These ratios would inhibit the activity of pyruvate dehydrogenase and thereby favour the conversion of pyruvate into lactate. This could account for the increased levels of lactate produced at the higher concentrations of indomethacin. However, indomethacin at a lower concentration of 0.1 mM did not have a significant effect on lactate production by rat jejunum in vitro. There are few reported studies of in vivo concentrations of
indomethacin in intestinal tissue after oral dosing. Weissenborn et al. [17] found a peak of approx. 25 μM after oral dosing of rats with indomethacin at concentrations that cause small intestinal ulcers. This is well below the concentrations found to affect mitochondria in the present study. However, the data of Weissenborn et al. [17] do not exclude the possibility of locally or transiently higher concentrations within the small intestine.

The effect of a pure uncoupler might not be expected to alter lactate production significantly. The trend for lactate levels produced by rat jejunal tissue to increase with treatment with DNP therefore appears paradoxical. However, DNP may cause inhibition of pyruvate transport into mitochondria, thereby favoring its conversion into lactate. A pH gradient has been shown to be essential for pyruvate to be transported into the matrix to enter the citric acid cycle [18–20]. The presence of an uncoupler, such as DNP, dissipates this pH gradient by acting as a protonophore that allows H⁺ ions to re-enter the mitochondria. The dissipation of this gradient would retard pyruvate transport into the mitochondria, leading to its accumulation in the cytoplasmic compartment and its subsequent conversion into lactate. This could account for the increased lactate levels produced by the tissue in the presence of DNP in our studies and others [21]. In addition, this effect could also blunt the increase in oxygen consumption. An alternative explanation could be that DNP undergoes metabolism in vivo to compounds with distinctive properties [22].

Both killed and anaesthetized animals were used to supply tissue for the experiments, to ascertain whether the use of anaesthetic agents affects the parameters measured. Studies have reported that anaesthesia per se affects energy metabolism in experimental animals [23,24]. In the present study, the findings in the tissue taken from anaesthetized and killed rats were broadly similar. However, the tissue from anaesthetized rats resembled the human tissue more closely than did that from killed rats. Differences in effects on energy charge between human and rat tissue may also reflect the presence of muscularis mucosa in the rat tissue, as this is not present in the human biopsies.

In conclusion, indomethacin affects mitochondrial function and hence energy metabolism in rat jejunal tissue in vitro at high concentrations of the drug. The findings in human jejunal mucosa in vitro are less conclusive, with effects seen on oxygen uptake, but not on lactate production or energy charge. The alterations in ATP level seen in the present study are not severe enough to trigger necrotic cell death [25], suggesting that apoptotic pathways may be significant in generating tissue damage following NSAID treatment. A full understanding of the processes triggered by NSAID treatment may make it possible to prevent the intestinal damage associated with the use of these drugs.

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REFERENCES


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