Effects of indomethacin on energy metabolism in rat jejunal tissue in vivo

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ABSTRACT

The non-steroidal anti-inflammatory drugs (NSAIDs) are a widely used group of drugs in clinical medicine. However, their propensity to cause gastrointestinal damage limits their clinical utility. The pathogenesis of this toxicity is not well established. It has been postulated that an early event in the development of damage is an effect of these drugs on mitochondrial function. The present paper sets out to evaluate the effects of indomethacin, a commonly used NSAID, on energy metabolism in vivo. Indomethacin was administered to male Sprague–Dawley rats, either intrajejunally or orally, and indices of mitochondrial function were determined. The parameters chosen for this purpose were oxygen uptake by, lactate levels in and the energy charge of jejunal tissue. Oxygen uptake by and energy charge in jejunal tissue were unaffected at 1 and 3 h after dosing by gavage with indomethacin. The drug significantly affected the tissue lactate/pyruvate ratio at 3 h (but not at 1 h) after oral dosing. Effects of indomethacin on jejunum incubated ex vivo were found to be reversible. The data suggest that indomethacin affects mitochondrial function in vivo, but that compensatory changes in glycolytic rate maintain energy charge.

INTRODUCTION

The non-steroidal anti-inflammatory drugs (NSAIDs) are used extensively in clinical medicine. However, they show a propensity to cause gastrointestinal ulceration and subsequent bleeding [1,2]. NSAIDs also cause an enteropathy, but the pathogenesis of this damage is controversial. NSAIDs are known to inhibit cyclooxygenase, but this does not fully explain the mechanism of damage, as it is possible to inhibit gastric cyclooxygenase almost completely without apparent damage [3]. It is also known that several NSAIDs, such as aspirin and indomethacin, act as uncouplers of mitochondrial oxidative phosphorylation [4]. Studies with these drugs in isolated mitochondrial preparations have shown that they uncouple oxidative phosphorylation at low concentrations and inhibit it at higher concentrations [5–7]. In addition, indomethacin has been shown to inhibit oxidative phosphorylation at high concentrations in jejunal tissue in vitro [8]. Evidence for mitochondrial damage in vivo has also been found, but this has been limited to morphological studies [6].

The present study was therefore undertaken in an attempt to determine whether the effects of indomethacin on rat jejunal tissue ex vivo and in vivo are similar to those suggested from in vitro studies [8], and to investigate the possible functional consequences, namely whether indomethacin leads to changes in ATP level/energy charge in vivo.

MATERIALS AND METHODS

Animals

Male Sprague–Dawley rats, 200–250 g body weight were obtained from Charles River UK Ltd, and used as the source of rat jejunal tissue. The rats were anaesthetized using an anaesthetic cocktail consisting of Hypnovel (midazolam hydrochloride 5 mg/ml; Roche) and Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone

Key words: apoptosis, ATP synthesis, glycolysis, non-steroidal anti-inflammatory drugs, oxidative phosphorylation, rat.
Abbreviations: DNP, 2,4-dinitrophenol; NSAID, non-steroidal anti-inflammatory drug.
Correspondence: Dr Robert J. Simpson (e-mail robert.simpson@kcl.ac.uk).
Determination of the effects of indomethacin on oxygen uptake and lactate production by rat jejunum ex vivo

Rats were fasted overnight. The next morning, they were dosed by oral gavage with indomethacin at 20 mg/kg or an equal volume of vehicle. This dose of indomethacin is high compared with the therapeutic dose in humans, but is a commonly used dose in intestinal toxicity studies in the rat. After 1 h, the rats were anaesthetized using the anaesthetic cocktail. A segment of mid-jejenum was removed, weighed (30–50 mg) and incubated at 37 °C in a Clarke’s oxygen electrode to measure oxygen uptake by the tissue. The buffer used in the incubation was: 125 mM NaCl, 3.5 mM KCl, 16 mM Hepes, 10 mM MgCl₂, 1 mM CaCl₂, 10 mM glucose, pH 7.4.

The amount of lactate released into the incubation medium by the tissue was measured as follows: 100 μl of the buffer was sampled at the end of the incubation period and assayed for lactate according to the spectrophotometric method of Beutler [9].

The experiment was repeated in another group of rats, from which jejunal tissue was obtained in the same fashion as described above, but 3 h after the oral dose of indomethacin or vehicle.

Determination of the effects of indomethacin and DNP on energy charge in rat jejunum in vivo

The drug was administered either intrajejunally or by gavage after an overnight fast. Intrajejunal injection was performed on rats anaesthetized using the anaesthetic cocktail. A laparotomy was performed and a loop of jejunum, 10 cm distal to the ligament of Treitz, was exposed. Indomethacin or DNP in 1 ml, or an equal volume of vehicle (10% DMSO), was injected into the jejunal lumen and left for 0.5, 5 or 60 min. Care was taken to see that the injected fluid did not flow proximally. The site of the injection was marked with a loose suture. At the end of the period, the luminal contents were flushed out without overdistending the jejunal loop, and tissue 5 cm distal to the site of injection was freeze-clamped. The flattened segment of tissue within the clamp was ground up in liquid nitrogen and thawed in 10% (v/v) perchloric acid. The resultant perchloric acid extract was centrifuged at 4 °C and neutralized with 3 M potassium carbonate, using 0.05% Methyl Orange as indicator. This extract was used for the determination of energy charge as described below.

In a separate series of experiments, rats were dosed orally with indomethacin (20 mg/kg) in 10% (v/v) DMSO. Control animals received an equal volume of 10% (v/v) DMSO. At 1 or 3 h later, the animals were anaesthetized using the anaesthetic cocktail. A laparotomy was then performed and jejunal tissue, approx. 15 cm distal to the ligament of Treitz, was isolated. This segment was freeze-clamped in vivo and used for assay of the adenine nucleotides and determination of the energy charge, as described above.

Determination of energy charge and lactate/pyruvate ratio

Lactate and pyruvate levels were measured in the neutralized extract according to the spectrophotometric method of Beutler [9]. Lactate/pyruvate ratios were then calculated. The neutralized extract obtained as detailed above was used in assays for the three adenine nucleotides, ATP, ADP and AMP, according to the spectrophotometric method of Beutler [9], and the energy charge [10] for each sample was calculated using the formula:

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

Determination of the effect of preincubation with indomethacin on oxygen uptake by rat jejunum in vitro

In order to determine whether the inhibitory effect of indomethacin on oxygen uptake by rat jejunum is reversible or irreversible, jejunal tissue was obtained from an anaesthetized rat and the baseline oxygen uptake was measured in the oxygen electrode, using Hepes buffer, pH 7.4, as described above. After this, the fragment was removed and incubated in buffer containing 2.5 mM indomethacin for 5 min. At the end of this period, the tissue was removed, rinsed briefly in fresh buffer and then returned to the oxygen electrode to measure oxygen uptake. The rate of uptake after incubation with the drug was compared with the rate before incubation. Control measurements were also carried out, as described above, with 10% (v/v) DMSO substituted for the drug.

Statistics

Student’s t test was used for statistical analysis of the data obtained. A P value of < 0.05 was considered significant.

RESULTS

No significant effects of orally administered indomethacin were seen on oxygen uptake or lactate pro-
Lactate production (nmol (20 mg value of 1 in the presence of the drug, compared with a control fall in oxygen uptake, i.e. 1 jejunal tissue contrast with results obtained when oxygen uptake by been preincubated with vehicle (Table 2). This was in uptake by the tissue when compared with tissue that had been preincubated with indomethacin (20 mg/kg) treatment (Table 1). Preincubation of tissue in ex vivo production by jejunal tissue ex vivo 1 and 3 h after the oral dose of the drug (Table 1). Preincubation of tissue in 2.5 mM indomethacin did not significantly affect oxygen uptake by the tissue when compared with tissue that had been preincubated with vehicle (Table 2). This was in contrast with results obtained when oxygen uptake by jejunal tissue in vitro was measured in the presence of 2.5 mM indomethacin. In this case there was a significant fall in oxygen uptake, i.e. 1.32 (±0.09) nmol·min⁻¹·mg⁻¹ in the presence of the drug, compared with a control value of 1.82 (±0.21) nmol·min⁻¹·mg⁻¹. The effect of intrajejunally administered indomethacin (20 mg/kg) on the energy charge of the jejunum was determined at various time intervals after injection. As shown in Table 3, there were no significant differences between the control and indomethacin-treated groups. However, within the control or drug-treated groups themselves, the energy charge measured at 60 min was significantly lower than that at 0.5 min.

Table 1: Effects of indomethacin on oxygen uptake and lactate production by rat jejunum ex vivo

Rats were dosed orally with equal volumes of either control solution (10% DMSO) or one containing indomethacin (20 mg/kg) after an overnight fast. They were then allowed free access to food and water. After 1 or 3 h, a fragment of jejunum was removed from each animal, under anaesthesia, and used for measurement of oxygen uptake and lactate production. Data are means (S.E.M.) for n = 6 rats in each group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>O₂ uptake (nmol·min⁻¹·mg⁻¹)</th>
<th>Lactate production (nmol·5 min⁻¹·mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h after dose</td>
<td>3 h after dose</td>
</tr>
<tr>
<td>Control (10% DMSO)</td>
<td>2.68 (0.39)</td>
<td>1.77 (0.14)</td>
</tr>
<tr>
<td>Indomethacin (20 mg/kg)</td>
<td>2.39 (0.18)</td>
<td>1.73 (0.17)</td>
</tr>
</tbody>
</table>

Table 2: Oxygen uptake by rat jejunum in vitro after preincubation with 2.5 mM indomethacin

Oxygen uptake by rat jejunal fragments was measured before and after incubation in 2.5 mM indomethacin or control solution (10% DMSO). Data are means (S.E.M.); n = 6.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Oxygen uptake at baseline (nmol·min⁻¹·mg⁻¹)</th>
<th>Oxygen uptake after treatment (nmol·min⁻¹·mg⁻¹)</th>
<th>% of uptake at baseline</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.42 (0.19)</td>
<td>3.31 (0.14)</td>
<td>139 (16)</td>
</tr>
<tr>
<td>Indomethacin (2.5 mM)</td>
<td>2.16 (0.13)</td>
<td>2.65 (0.12)</td>
<td>123 (2)</td>
</tr>
</tbody>
</table>

Table 3: Effects of indomethacin (20 mg/kg) on energy charge of rat jejunum in vivo at various time intervals after intrajejunal administration of the drug

Animals were anaesthetized, then segments of jejunum, 15 cm distal to the ligament of Treitz and 5 cm distal to site of intrajejunal injection, were freeze-clamped at 0.5, 5 and 40 min after intrajejunal injection of indomethacin or control solution. Energy charge was determined in these segments. Data are means (S.E.M.); n = 6. Significance of differences: *P < 0.05, **P < 0.01 when energy charge at 60 min was compared with that at 0.5 min, using Student’s t test.

<table>
<thead>
<tr>
<th>Energy charge</th>
<th>0.5 min</th>
<th>5 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.88 (0.02)</td>
<td>0.79 (0.04)</td>
<td>0.77 (0.02)**</td>
</tr>
<tr>
<td>Indomethacin (20 mg/kg)</td>
<td>0.87 (0.02)</td>
<td>0.83 (0.02)</td>
<td>0.79 (0.03)*</td>
</tr>
</tbody>
</table>

Table 4: Effects of indomethacin and DNP on energy charge and lactate/pyruvate ratio in vivo, 5 min after intrajejunal injection of the drug

Segments of jejunum, 15 cm distal to the ligament of Treitz and 5 cm distal to site of intrajejunal injection, were freeze-clamped at 5 min after intrajejunal injection of indomethacin, DNP or control solution. These segments were used for the determination of energy charge and lactate/pyruvate ratio. Data are means (S.E.M.); n = 6. Significance of difference: *P < 0.05 compared with control, using Student’s t test.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Energy charge</th>
<th>Lactate/pyruvate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.80 (0.01)</td>
<td>4.63 (0.65)</td>
</tr>
<tr>
<td>Indomethacin 0.1 mM</td>
<td>0.79 (0.02)</td>
<td>3.52 (0.65)</td>
</tr>
<tr>
<td>Indomethacin 0.5 mM</td>
<td>0.79 (0.01)</td>
<td>3.23 (0.42)</td>
</tr>
<tr>
<td>Indomethacin 2.5 mM</td>
<td>0.81 (0.01)</td>
<td>3.9 (0.63)</td>
</tr>
<tr>
<td>DNP 0.1 mM</td>
<td>0.71 (0.03)*</td>
<td>4.41 (0.60)</td>
</tr>
</tbody>
</table>

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DISCUSSION

Ultrastructural damage suggestive of uncoupling of
intestinal mitochondria has been shown to occur as early
as 1 h after an oral dose of indomethacin, with more
severe changes evident 3 h after oral dosing [6]. The
rationale behind choosing to measure the various para-
eters of energy metabolism, i.e. oxygen uptake,
lactate production and energy charge, at 1 and 3 h after
the administration of the drug was to allow a direct
comparison with previous studies. Like most other
NSAIDs, indomethacin undergoes an enterohepatic cir-
culation, which may be relevant for the 3 h data. The
concentrations of indomethacin present in jejunal cells
in vivo
1 and 3 h after an oral dose are not known with
certainty. Weissenborn et al. [11] showed that, 1 h after
an oral dose of indomethacin (10 mg/kg), the concen-
tration of the drug in the jejunal mucosa was approx.
0.01 mM, while the level after 3 h was approx. 0.02 mM.
In the present study, the oral dose of indomethacin used
was 20 mg/kg. Based on the data of Weissenborn et al.
[11], which do not take into account the possible
subcellular compartmentalization of drug distribution,
this dose of drug would result in concentrations well
below those shown to produce uncoupling or inhibition
in mitochondrial preparations and in tissue in vitro [5–7].
This appears to be borne out by the observations that no
significant effects of the drug were demonstrable on the
mitochondrial parameters measured after the oral dose
(Tables 1 and 5). The only indication that energy
metabolism in the tissue may have been affected lay in the
finding that the lactate/pyruvate ratio was significantly
higher in the indomethacin-treated tissue compared with
control tissue, indicating an accelerated rate of glycolysis
(Table 5).

Another possibility that might explain a lack of
demonstrable effects is that the effects of indomethacin
may be reversible, and hence it may not be possible to
detect any in vivo changes in the tissue using ex vivo
measurements. In order to test this hypothesis, jejunal
tissue obtained from an anaesthetized rat was preincu-
bated for 5 min with indomethacin at a concentration of
2.5 mM, which is known to be inhibitory [8], after
measurement of its baseline rate of oxygen uptake. The
tissue was then transferred back to the oxygen electrode
to measure its rate of oxygen uptake again, in indo-
methacin-free medium. There was no significant dif-
ference between the rates of uptake by tissue that had
been incubated in 2.5 mM indomethacin and that which
had been incubated in control solution (Table 2). This
seems to indicate that the inhibition produced by 2.5 mM
indomethacin is reversible. Once the tissue is transferred
out of the indomethacin solution into incubation buffer,
the drug within the tissue probably diffuses out of the
cells into the medium, and the levels within the enterocy-
tes would then no longer be high enough to sustain any
effect that may have occurred. The reversible nature of
the inhibition produced by indomethacin in jejunal tissue
in vitro could also account for the findings that no effects
of the drug were demonstrable on oxygen uptake and
lactate production measured ex vivo
1 and 3 h after the
oral dose (Table 1).

The findings in Table 3 show that the energy charge of
mucosal integrity and cellular energy levels [15].
Thus determinations of parameters of energy metabolism

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Energy charge</th>
<th>Lactate/pyruvate ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 h after dose</td>
<td>3 h after dose</td>
</tr>
<tr>
<td>Control</td>
<td>0.87 (0.01)</td>
<td>0.85 (0.01)</td>
</tr>
<tr>
<td>Indomethacin (20 mg/kg)</td>
<td>0.88 (0.01)</td>
<td>0.87 (0.01)</td>
</tr>
</tbody>
</table>

The energy charge of the tissue was unaffected at both
1 and 3 h after an oral dose of indomethacin (Table 5).
The lactate/pyruvate ratio was unchanged at 1 h, but was
raised significantly at 3 h.
in vivo, if done under the influence of anaesthesia (as they invariably are), might tend to give underestimates of the true values. This poses particular problems for the interpretation of such data. Under these circumstances, small falls in energy charge (as are likely to be produced by indomethacin) may not be detectable. This may account for the finding that changes in energy charge in vivo were not demonstrable in indomethacin-treated rats.

Similar concentrations of indomethacin that were used for in vitro incubations [8], i.e. 0.1, 0.5 and 2.5 mM, were next injected intrajejunally to make direct comparisons of effects in vitro and in vivo. There was no demonstrable effect of indomethacin on energy charge in this instance either (Table 4). Reasons similar to those outlined above may account for these findings. It is noteworthy, however, that DNP had a detectable effect, presumably reflecting the greater uncoupling potency of DNP as compared with indomethacin [7].

These observations show that it is difficult to demonstrate the effects of indomethacin on energy charge in vivo. In addition, the problem of relating in vitro inhibition and in vivo effects is highlighted. The observation that indomethacin was found to perturb energy metabolism in vitro [8] is not sufficient evidence that similar effects occur in vivo, for a variety of reasons. The compositions of in vitro incubation mixture may vary more widely than those present in the cells of the body. The relative proportions of enzymes, substrates, cofactors, the pH and ionic constituents of the incubation media may bear little resemblance to physiological requirements [16]. The concentration of a drug in vitro may vary with both tissue and time in the body, whereas in vivo it will remain relatively constant. It is not possible to predict accurately the tissue concentration of a drug from a knowledge of the exact size of the ingested dose, since, in individual animals or subjects, factors such as absorption from the gut and subsequent distribution among the various body fluids, organs and tissues vary widely [16].

A direct demonstration of an uncoupling or inhibitory action of indomethacin in the whole animal, in terms of its effects on energy metabolism, may therefore be difficult to accomplish. Studies have shown that administration of NSAIDs, such as aspirin and sodium salicylate, did not decrease either the normal ATP concentration in the liver and paw, or the elevated ATP concentrations in oedematous paws, in rats [17–19]. On the other hand, some studies have shown the opposite effect on energy metabolism. Jorgensen et al. [20] and Arvanitakis et al. [21] measured falls in ATP levels in pig gastric mucosa and human jejunal biopsies respectively after ingestion of aspirin. However, the doses used in these studies were very high, 10 g in the former and 2.6 g in the latter, both administered as a single dose. These large doses are probably responsible for the effects seen on ATP levels when measurements were made ex vivo.

The most probable explanation for the lack of effect of indomethacin in vivo is that, unlike isolated mitochondria and jejunal tissue in vitro, the whole animal seems to be able to maintain ATP levels in the presence of NSAIDs such as indomethacin and aspirin (at pharmacological doses). Greater amounts of substrate can be sacrificed to supply energy and to maintain energy levels. In addition, ATP may be formed by anaerobic phosphorylation reactions at the substrate level, which are not affected by these drugs. This has been demonstrated in an in vitro system by Kalbhen and Domenjoz [18]. They showed that when neoplastic mast cells, which rely on glycolysis for most of their energy production, are incubated with salicylate, the level of ATP increases, although the efficiency of ATP production by the respiratory chain is reduced. The present study also documented an increased rate of glycolysis in indomethacin-treated tissue. It must also be kept in mind that most NSAIDs are protein-bound in the circulation (> 95%), and there may be insufficient levels of free drug to exert uncoupling effects. Another possible explanation for the lack of effect of indomethacin in vivo is the occurrence of proton leak in oxygen-consuming tissues of the rat [22,23]. Proton leak is responsible for a significant proportion of total oxygen uptake by the tissue. It could thus account for the observation that no changes occurred in energy charge in vivo, as the mitochondria may be already partially uncoupled in vivo. Effects of indomethacin on mitochondrial function in vivo and ex vivo are difficult to demonstrate. Thus it may not be possible to detect small degrees of impairment of mitochondrial function that may, nevertheless, be detrimental to the tissue.

In conclusion, only minor changes in intestinal energy levels occur after indomethacin dosing in vivo. Recent advances have increased our understanding of mechanisms of cell death and how these relate to energy metabolism and mitochondrial function [24]. In particular, it has been shown that small decreases in cellular ATP levels are associated with apoptotic cell death, if accompanied by appropriate triggers. Mitochondrial damage has been shown to be a trigger for apoptosis [24]. This information, combined with previous evidence for mitochondrial uncoupling in vivo [6], suggests that indomethacin does not induce conditions likely to produce widespread necrotic cell death; however, apoptosis may be triggered by the mitochondrial damage [25].

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