

# Protective effect of metronidazole on uncoupling mitochondrial oxidative phosphorylation induced by NSAID: a new mechanism

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## Abstract

**Background**—The pathogenesis of non-steroidal anti-inflammatory drug (NSAID) enteropathy is complex. It involves uncoupling of mitochondrial oxidative phosphorylation which alters the intercellular junction and increases intestinal permeability with consequent intestinal damage. Metronidazole diminishes the inflammation induced by indomethacin but the mechanisms remain speculative. A direct effect on luminal bacteria has traditionally been thought to account for the protective effect of metronidazole. However, a protective effect of metronidazole on mitochondrial oxidative phosphorylation has never been tested.

**Aims**—To assess the protective effect of metronidazole on mitochondrial uncoupling induced by indomethacin and also on the increased intestinal permeability and macroscopic damage.

**Material and methods**—The protective effect of metronidazole was evaluated in rats given indomethacin; a macroscopic score was devised to quantify intestinal lesions, and intestinal permeability was measured by means of <sup>51</sup>Cr-ethylenediaminetetraacetic acid. The protective effect of metronidazole against mitochondrial uncoupling induced by indomethacin was assessed using isolated coupled rat liver mitochondria obtained from rats pretreated with metronidazole or saline.

**Results**—Metronidazole significantly reduced the macroscopic intestinal damage and increase in intestinal permeability induced by indomethacin; furthermore, at the mitochondrial level, it significantly reduced the increase in oxygen consumption in state 4 induced by indomethacin and caused less reduction of the respiratory control rate.

**Conclusion**—Our study confirmed the beneficial effects of metronidazole on intestinal damage and intestinal permeability, and demonstrated, for the first time, a direct protective effect of metronidazole on uncoupling of mitochondrial oxidative phosphorylation caused by NSAIDs.

(Gut 2001;48:163-167)

Keywords: uncoupling agents; intestinal permeability; enteropathy; non-steroidal anti-inflammatory drugs; metronidazole; indomethacin; rats

Non-steroidal anti-inflammatory drugs (NSAIDs) have been used widely because of their analgesic, anti-inflammatory, and antipyretic properties. The main concern with these drugs is the frequency and severity of their digestive side effects,<sup>1</sup> involving the entire gastrointestinal tract.<sup>2,3</sup> Scintilograms suggest small intestinal inflammation in 42% of patients taking NSAIDs, faecal calprotectin levels suggest inflammation in 44%,<sup>4</sup> but when faecal excretion of <sup>111</sup>In-labelled leucocytes was used as a measure of inflammation, up to 67%<sup>5-9</sup> of patients had an enteropathy. Similarly, intestinal lesions detected by enteroscopy<sup>10</sup> have been described in 66% of patients and in a postmortem study, the prevalence of non-specific intestinal ulceration was 13.5% in those who had consumed NSAIDs for a long period of time (six months or more).<sup>11</sup> The intestinal lesions caused by NSAIDs may lead to chronic bleeding,<sup>12-14</sup> protein loss<sup>8</sup> and, occasionally, strictures.<sup>15-20</sup> There have also been reports of NSAIDs causing enteritis and/or colitis,<sup>21-27</sup> decreased xylose absorption,<sup>28</sup> and increased incidence of perforation,<sup>29</sup> and also associations with diverticulitis,<sup>30,31</sup> appendicitis,<sup>32</sup> internal fistulas,<sup>33</sup> and relapse of inflammatory bowel disease.<sup>34-36</sup>

The pathogenesis of NSAID enteropathy is complex and there are many uncertainties. Somasundaram and colleagues<sup>37</sup> suggest that there are three crucial steps in the pathogenesis. The *first step* involves specific biochemical damage of mitochondria,<sup>38-40</sup> uncoupling the oxidative phosphorylation reaction, during drug absorption and/or after biliary excretion (enterohepatic circulation).<sup>41-45</sup> Electron microscopic studies show vacuolisation and ballooning of mitochondria within an hour of indomethacin administration which is highly characteristic of uncoupling of oxidative phosphorylation.<sup>46</sup> The consequence of uncoupling is diminished cellular ATP, which alters the intercellular junction, increases intestinal permeability, and releases calcium into cytosol which in turn causes secondary biochemical damage.<sup>9,47-50</sup> NSAID inhibition of cyclooxygenase appears not to be involved in this

**Abbreviations used in this paper:** NSAIDs, non-steroidal anti-inflammatory drugs; DMSO, dimethyl sulphoxide; <sup>51</sup>Cr-EDTA, <sup>51</sup>Cr-ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; HEPES, N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic acid); RCR, respiratory control rate.

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Accepted for publication  
22 August 2000

framework.<sup>51–52</sup> In the context of NSAID induced enteropathy, increased intestinal permeability (*transitional stage*) will convert the biochemical damage into a tissue reaction. In the *second step*, mucosa is exposed to digestive enzymes and bile,<sup>47–48</sup> and bacteria<sup>53–58</sup> and their products, which appear to be the main neutrophil chemoattractants.<sup>59–61</sup> When phagocytosis takes place, neutrophils may cause tissue damage by free radical<sup>62–63</sup> production and lysosomal release. The role of bacteria has been inferred by almost universal findings which have demonstrated that indomethacin provokes very few macroscopic lesions in germ free animals<sup>56–57</sup> and in those pretreated with antibiotics.<sup>53–55–64</sup>

Metronidazole seems to diminish most parameters of inflammation induced by indomethacin; nevertheless, the mechanism by which the drug protects the mucosa and attenuates inflammation remains only speculative. Data from the medical literature suggest that metronidazole may have a direct effect on luminal bacteria attenuating enhanced mucosal permeability caused by indomethacin and reducing massive bacterial translocation into the mesenteric lymph nodes, liver, and spleen,<sup>65</sup> rather than a cytoprotective role. In this context, this drug should protect only during the *second step*, where endogenous bacteria are involved, although in some studies metronidazole protected against the increase in intestinal permeability induced by NSAIDs, indicating a direct effect of metronidazole at the beginning of intestinal damage. Bjarnason and colleagues<sup>64</sup> reported that intestinal inflammation and blood loss were significantly reduced with metronidazole; nevertheless they were unable to show a significant difference in intestinal permeability after treatment with metronidazole. However, this work included patients who had been taking NSAIDs for at least six months. Accordingly, Yamada and colleagues,<sup>65</sup> in an experimental model, found that metronidazole did not reduce mucosal permeability at one day following injection of indomethacin but mucosal permeability was reduced after 48 hours, and concluded that metronidazole probably had a direct effect on the late stage (*second step*) involving luminal bacteria. In contrast, Davies and colleagues,<sup>66</sup> in humans, showed that coadministration of metronidazole for a short period of time successfully prevented the indomethacin induced change in intestinal permeability. Similarly, Davies and Jamali,<sup>67</sup> in rats, demonstrated that metronidazole reversed the increased intestinal permeability caused by NSAIDs but attributed this finding to the potential free radical scavenger action of metronidazole. Thus the exact point(s) where metronidazole protects small intestinal injury is(are) not known. The finding of precocious metronidazole protection is important because it would indicate a direct effect of the drug at the beginning of NSAID injury. To test this hypothesis, we have studied the protective effect of metronidazole on macroscopic damage and intestinal permeability induced by indomethacin in rats and in isolated coupled mitochondria in an attempt to determine the mechanism

underlying metronidazole protection of NSAID induced small intestinal injury.

### Material and methods

Male Wistar rats (250–350 g) were housed in individually metabolic cages. Rats were given water and standard laboratory rat chow ad libitum. Eighty rats were divided into three groups: vehicle (water) control group (n=27), indomethacin group (n=26), and indomethacin in combination with metronidazole group (n=27).

Intestinal inflammation was induced by administration of a single dose of indomethacin (7.5 mg/kg) by gavage. The drug was initially dissolved in dimethyl sulphoxide (DMSO) and diluted so that the final concentration of DMSO was 5 % (v/v) and adjusted to pH 7.4, after which they had free access to standard rat food and water.

Metronidazole was given by gavage in three divided doses (60 mg/kg/dose—total dose 180 mg/kg), 12 hours apart, beginning at the same time as indomethacin.

Intestinal permeability was assessed using urinary excretion of <sup>51</sup>Cr-ethylenediaminetetraacetic acid (<sup>51</sup>Cr-EDTA), as previously described,<sup>48</sup> after oral administration. Animals received either indomethacin and/or metronidazole as described above. Rats were given 5  $\mu$ Ci of <sup>51</sup>Cr-EDTA in 0.5 ml of distilled water by gavage, followed by 5 ml of water. Animals were then placed in individual metabolic cages for five hours for collection of urine and had free access to tap water and food. Rats were sacrificed by lethal injection (50 mg) of ketamine (Ketalar) and laparotomy was performed and the bladder emptied by puncture. Total five hour radioactivity excreted in urine was determined together with standards in a gamma counter for two minutes. Data were expressed as fractional excretion of the radioactive marker. The small intestine was then gently removed, the intestinal mucosa was exposed by cutting through the contra mesenteric side, laid out on a piece of cork, and dried with Evan's blue to improve assessment of macroscopic score, 29 hours after giving the drugs, using a stereostatic microscope (25–50 times). A macroscopic score was devised to quantify intestinal lesions: total number of mucosal ulcers with: (a) <1 mm; (b)  $\geq$ 1 and <3 mm; (c)  $\geq$ 3 and <5 mm; (d)  $\geq$ 5 and <10 mm; and (e)  $\geq$ 10 mm, multiplied by 1, 3, 5, 10, and 20, respectively. Total score was the sum of the values obtained in each item. A separate group of male Wistar rats (n=18) weighing 250 g was used to determine the effect of indomethacin on mitochondria in vitro, and was subdivided into two groups: one received a single dose of 100 mg of metronidazole intraperitoneally and the other saline, both five hours before liver extraction and mitochondrial isolation.

Preparation of coupled mitochondria was as previously described<sup>68</sup> from animals sacrificed by decapitation. The liver was rapidly dissected and placed in ice cold homogenising solution No 1 (280 mM sucrose, 0.1 mM EGTA (ethylene glycol-bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid), 2 mM HEPES (N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulphonic

acid)), 5 mg/ml bovine serum albumin at pH 7.4), cut finely into approximately 1 cm pieces with scissors, and washed twice with homogenising solution to remove excess blood. The liver was then suspended in 50 ml of the same homogenising solution and homogenised in a Potter-Elvehjem homogeniser by six strokes with a rotation Teflon pestle. The homogenate was then centrifuged at 900 *g* for 10 minutes to remove excess blood, nuclei, and cell debris. The supernatant was centrifuged at 10 000 *g* for another 10 minutes, after which the pellet was removed and resuspended in 10 ml of homogenising solution No 2 (280 mM sucrose, 2 mM HEPES, pH 7.4). The isolation procedure took approximately 45 minutes with the homogenate being kept at 0–4°C. Mitochondrial protein concentration was determined using Lowry's method.<sup>69</sup>

Oxygen consumption was polarographically measured<sup>70</sup> using a Clarke type oxygen electrode (Clark; Yellow Springs Instruments Co., Yellow Springs, Ohio, USA) as described by Chance and Williams.<sup>71</sup> The electrode was fitted into a thermostatic Plexiglas chamber containing 1.55 ml of oxygen electrode buffer (120 mM KCl, 5 mM de Tris, 1 mM EGTA, bovine serum albumin 0.1%, pH 7.4). A small amount (100 µl) of the mitochondrial preparation, 35 µl of potassium succinate (10 mM), 20 µl of ADP (504 mM), and 0–100 µl of indomethacin (to a final concentration of 0–140 µmol/mg protein) were introduced through a small hole in the chamber lid. The experiments were carried out at 30°C with continuous magnetic stirring. Oxygen consumption was measured after five minutes and monitored for approximately two minutes for each experiment. Three to six experiments were performed at each indomethacin concentration for each rat. Indomethacin was dissolved in DMSO (final concentration of DMSO in the chamber never exceeded 0.3% v/v). Control experiments used distilled water only as in a prior experiment conducted in our laboratory we verified that DMSO, in concentrations up to 10 times higher than the maximum used in the present *in vitro* experiments, was similar to water in terms of oxygen consumption.

#### STATISTICAL ANALYSIS

Results are expressed as mean (SEM). Statistical differences in macroscopic scores and intestinal permeability among groups were assessed using non-parametric tests (Kruskal-Wallis and Dunn). Statistical differences in oxygen consumption between DMSO and water, and controls in the metronidazole and water groups were assessed by a non-parametric test (Mann-Whitney). Statistical differences in oxygen consumption between groups were assessed by regression analysis.

## Results

### MACROSCOPIC DAMAGE

Figure 1 shows that the values for macroscopic scores were significantly different among the three groups: control, indomethacin, and indomethacin plus metronidazole groups ( $p < 0.0001$ ). Using a discriminatory test it was

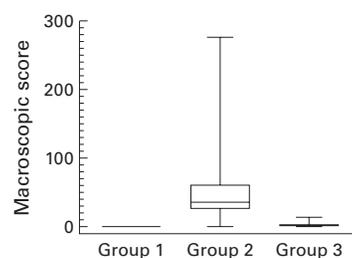


Figure 1 Box and whisker plot showing median macroscopic score (horizontal line), 25th and 75th centiles (box), and range (whiskers) in the control (group 1,  $n=10$ ), indomethacin (group 2,  $n=10$ ), and indomethacin plus metronidazole (group 3,  $n=10$ ) groups. Group 1 *v* group 2,  $p < 0.0001$ ; group 1 *v* group 3, NS; group 2 *v* group 3,  $p < 0.001$ .

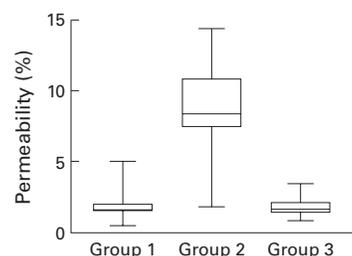


Figure 2 Box and whisker plot showing median intestinal permeability (horizontal line), 25th and 75th centiles (box), and range (whiskers) in the control (group 1,  $n=17$ ), indomethacin (group 2,  $n=16$ ), and indomethacin plus metronidazole (group 3,  $n=17$ ) groups. Values are percentage of  $^{51}\text{Cr-EDTA}$  excreted in five hour urine. Group 1 *v* group 2,  $p < 0.05$ ; group 1 *v* group 3, NS; group 2 *v* group 3,  $p < 0.05$ .

possible to see that macroscopic scores were significantly lower when metronidazole was administered together with indomethacin compared with indomethacin alone (indomethacin with metronidazole 3.3 (1.4) *v* indomethacin 63.6 (25.9);  $p < 0.05$ ) and was not different from the control group (indomethacin with metronidazole 3.3 (1.4) *v* control 0; NS).

### INTESTINAL PERMEABILITY

Intestinal permeability, as assessed by  $^{51}\text{Cr-EDTA}$ , was significantly different among the three groups ( $p < 0.0001$ ) (fig 2). Intestinal permeability was significantly increased after indomethacin (controls 2.0 (0.3)% *v* indomethacin 8.9 (0.8)%;  $p < 0.0001$ ). When metronidazole was added, there was no significant difference compared with controls (indomethacin with metronidazole 1.9 (0.2)%; NS), but it was significantly different from indomethacin alone ( $p < 0.05$ ).

### OXYGEN CONSUMPTION

The increase in oxygen consumption induced by indomethacin was significantly lower in the metronidazole group ( $p = 0.046$ ) (fig 3); in addition, there was a less pronounced reduction in respiratory control rate (RCR) ( $p = 0.035$ ) (fig 4) compared with the metronidazole group. There was no significant difference in oxygen consumption in state 4 (S4) (control 48.2 (2.3) *v* metronidazole 40.1 (3.1);  $p = 0.16$ ) or RCR (control 3.1 (0.1) *v* metronidazole 2.8 (0.1);  $p = 0.29$ ) between both groups before administration of indomethacin. These data are consistent with the results of Aicardi

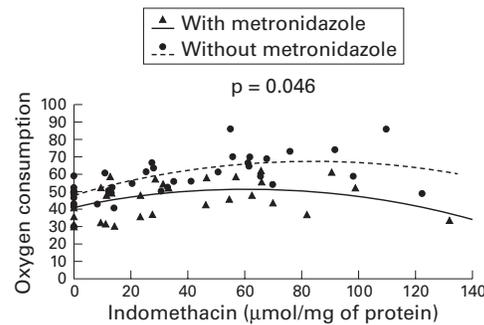


Figure 3 Regression analysis of the effects of metronidazole on oxygen consumption in the basal state (S4) (values are  $\mu\text{mol}$  of oxygen/mg of protein).

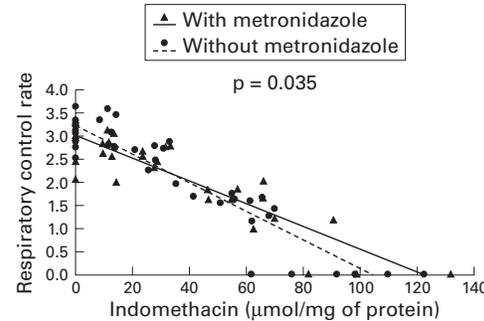


Figure 4 Regression analysis of the effects of metronidazole on respiratory control rate (RCR). Relation between consumption of oxygen with ADP (S3) and after it has been consumed (S4).

and Solaini<sup>72</sup> which showed no effect of metronidazole alone on oxygen consumption.

### Discussion

NSAID induced enteropathy includes three distinct phases<sup>37</sup>: an early phase (*first step*) involving uncoupling of mitochondrial oxidative phosphorylation followed by a *transitional stage* characterised by increased intestinal permeability, and a late phase (*second step*) when the intestinal mucosa is exposed to aggressive agents (for example, bacteria and their products) that promote the release of inflammatory mediators with ensuing additional increase in intestinal permeability and finally, tissue damage.

The protective effect of metronidazole on NSAID induced enteropathy has been studied recently.<sup>64–67</sup> However, the mechanisms by which metronidazole exerts this protection are not completely understood. Yamada and colleagues,<sup>65</sup> in a rat model of intestinal inflammation (indomethacin), suggested that the protective effect of metronidazole was due to its antibacterial action. However, Davies and Jamali,<sup>67</sup> using a similar experimental model, demonstrated that metronidazole protected against increased intestinal permeability induced by indomethacin at an early stage (12 hours after indomethacin) and concluded that metronidazole directly interfered at a time when participation of luminal bacteria was less evident.<sup>37</sup> Moreover, Davies and Jamali<sup>67</sup> showed that the protective effect of metronidazole was dose dependent. Methodological differences between these studies<sup>65, 67</sup> probably explain the discrepancies.

Our results confirm previous work<sup>65, 67</sup> where metronidazole was associated with a significant reduction in macroscopic intestinal damage induced by NSAIDs (fig 1). Also, metronidazole prevented NSAID induced permeability change (fig 2) approximately 24 hours after administration of indomethacin. In common with Davies and Jamali,<sup>67</sup> we also noticed a dose dependent relationship in metronidazole protection (data not shown). The intestinal damage induced by indomethacin reaches its maximal after 3–4 days<sup>55</sup> when luminal bacteria and their products have a place. The protection we observed with metronidazole, about 24 hours after indomethacin, suggests that metronidazole may intervene in an early phase of intestinal injury with little or no influence of luminal offensive agents. Also, a direct effect of metronidazole—and not an antibiotic mediated effect—is corroborated by the recent demonstration that metronidazole minimises indomethacin induced intestinal injury in germ free rats.<sup>73</sup> Our in vitro studies confirmed this hypothesis. Mitochondrial uncoupling is characterised by increased oxygen consumption in stage 4 (S4) and reduced RCR. Metronidazole significantly reduced the increase in mitochondrial oxygen consumption in stage 4 (S4) (fig 3) induced by indomethacin and caused less reduction in RCR (fig 4). Both features are related to a direct protective effect of metronidazole on uncoupling of mitochondrial oxidative phosphorylation caused by NSAID.

In the present work, we have described, for the first time, a new effect of metronidazole: its direct effect on mitochondrial oxidative phosphorylation and, indirectly, on the intercellular junction. Thus the protective effect of metronidazole against NSAID induced increased intestinal permeability may include an action on mitochondrial uncoupling. Our work uncovers new perspectives in the study of drugs that can directly interfere with mitochondrial uncoupling and eventually protect against NSAID induced enteropathy.

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