Aus der Klinik für Anästhesiologie
der Heinrich-Heine-Universität Düsseldorf
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Effect of indomethacin on hepatic and colonic mitochondrial function in tissue homogenates from healthy rats.

An experimental *in vitro* study.

Dissertation

zur Erlangung des Grades eines Doktors der Medizin der
Medizinischen Fakultät der Heinrich-Heine-Universität
Düsseldorf

vorgelegt von

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2021

Als Inauguraldissertation gedruckt mit der Genehmigung der Medizinischen Fakultät der Heinrich-Heine-Universität Düsseldorf

gezeichnet:
Dekan: Prof. Dr. med. Nikolaj Klöcker
Erstgutachter: Prof. Dr. med. Olaf Picker
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Dedicated to my always loving and supporting family.

Publication

Parts of this work have been published in:

Herminghaus A, Buitenhuis AJ, Schulz J, Truse R, Vollmer C, Relja B, Bauer I, Picker O. Indomethacin increases the efficacy of oxygen utilization of colonic mitochondria and uncouples hepatic mitochondria in tissue homogenates from healthy rats. Frontiers in Medicine. 2020;7:463

Summary

Indomethacin (INDO) belongs to the group of non-selective non-steroidal anti-inflammatory drugs (NSAIDs) and is known for its detrimental side effects on the lower gastrointestinal tract, like ulceration and bleedings, and for hepatotoxicity. From *in vivo* studies on small intestines from rats it is known that INDO exhibits substantial tissue injury (microvascular disorder, villus shortening, inflammation and epithelial disruption) due to mitochondrial uncoupling. Yet, there is no literature about the effects of INDO on colon and equivocal results regarding liver tissue. The aim of this study was to explore the mitochondrial function of rat colon and liver tissue under different doses of INDO *in vitro* for a possible explanation of the pathomechanisms on the adverse effects of INDO seen in colon and liver. Mitochondrial oxygen consumption was polarographically measured using a Clark type electrode in colon and liver homogenates from healthy rats after incubation with dimethyl sulfoxide (DMSO) (control) and INDO (diluted in DMSO) (colon: 0.36, 1, 30, 179, 300, 1000, 3000 µM INDO; liver: 0.36, 1, 3, 10, 30, 100, 179 µM INDO; n=6). Substrate-dependent (state 2) and ADP-dependent (state 3) respiration were computed. As an indicator for the coupling between the electron transport system (ETS) and oxidative phosphorylation (OXPHOS) the respiratory control index (RCI) was derived. The ADP/O ratio was calculated as an indicator of efficiency of OXPHOS. The data were non parametrically distributed therefore a Kruskal-Wallis test and a Dunn's correction were conducted. Differences in medians with $p < 0.05$ vs. control were considered significant and are presented as median (% of control) and interquartile range. INDO did not affect the RCI vs. control of colonic mitochondria. In colonic mitochondria the ADP/O ratio rose at concentrations of 1000 and 3000 µM INDO to 159 % (133 – 166 %) respectively to 183 % (167 – 349 %) and in complex II at concentrations of 179 and 3000 µM INDO to 142 % (113 – 150 %) respectively to 175 % (140 – 238 %). In hepatic mitochondria the RCI vs. control decreased for complex I and complex II at a concentration of 179 µM INDO to 41 % (32 – 50 %) respectively to 73 % (66 – 75 %). The ADP/O ratio vs. control was only altered in complex I triggered respiration at a concentration of 179 µM INDO to 994 % (674 – 1190 %). Summarizing the results, INDO affected colonic and hepatic mitochondria in an organ-specific, concentration-dependent and complex specific manner. Since INDO increased the efficiency of the OXPHOS in colonic cells, the deleterious GI complications associated with NSAID treatment are most likely not mediated via colonic mitochondria. On the other hand, the strong increase of the ADP/O ratio in hepatic mitochondria may reflect complex I specific terminal uncoupling leading to an energy depletion which could explain the pathomechanism of NSAIDs hepatotoxicity.

Zusammenfassung

Zahlreiche hepatische und gastrointestinale Nebenwirkungen nichtsteroidaler Antirheumatika (NSAR), wie z.B. Ulzerationen, Blutungen und Leberfunktionsstörungen, werden möglicherweise verursacht durch eine Störung der mitochondrialen Funktion mit schlussendlich kompromittierter ATP Synthese. Diese These wird vor allem für den Gastrointestinaltrakt durch *in vivo* Studien an gesunden Ratten gestützt, in welchen gezeigt werden konnte, dass sich mikrovaskuläre Störungen, Zottenveränderungen, Entzündungen und Epithelzerfall im Dünndarm unter der Gabe von NSAR entwickeln. In der Literatur gibt es allerdings keine Studien zur direkten Interaktion von NSAR mit der mitochondrialen Funktion von Colonzellen sowie widersprüchliche Ergebnisse zu Leberzellen. Ziel dieser Arbeit war es daher, die mitochondriale Funktion von Leber- und Colonzellen gesunder Ratten unter verschiedenen Konzentrationen von Indomethacin (INDO) zu untersuchen, um einen möglichen Erklärungsansatz für die oben beschriebenen Nebenwirkungen von NSAR zu finden. Der Sauerstoffverbrauch wurde in Colon- und Leberzellhomogenaten polarographisch mittels einer Clark-Elektrode nach Inkubation mit Dimethylsulfoxid (DMSO) (Kontrolle) oder INDO (in DMSO verdünnt) (Colon: 0,36, 1, 30, 179, 300, 1000, 3000 µM; Leber: 0,36, 1, 3, 10, 30, 100, 179 µM; n=6) und unter Gabe von Substrat (State 2) und ADP (State 3) gemessen. Als Indikator der Kopplung zwischen dem Elektronentransportsystem (ETS) und der oxidativen Phosphorylierung (OXPHOS) wurde der *Respiratory Control Index* (RCI) berechnet. Zur Bestimmung der Effizienz der OXPHOS wurde die ADP/O-Ratio bestimmt. Die erhobenen Daten waren nicht parametrisch verteilt, daher wurde ein Kruskall-Wallis-Test und Dunn's-Test durchgeführt. Signifikanzen von $p < 0,05$ werden im Weiteren dargestellt (Median in % der Kontrolle und Interquartilbereich). Der RCI vs. Kontrolle bestimmt in Colonzellhomogenaten, zeigte keine Veränderung in Komplex I und II unter Einwirkung von INDO. Die ADP/O-Ratio vs. Kontrolle gemessen in Colonzellhomogenaten für Komplex I stieg unter Einfluss von 1000 µM INDO auf 159 % (133 – 166 %) und unter 3000 µM INDO auf 183 % (167 – 349 %) sowie für Komplex II unter 179 µM INDO auf 142 % (113 -150 %) und unter 3000 µM INDO auf 175 % (140 – 238 %). In Leberzellhomogenaten wurde ein Absinken der RCI vs. Kontrolle unter der Wirkung von 179 µM INDO in Komplex I auf 41 % (32 – 50 %) und Komplex II auf 73 % (66 – 75 %) beobachtet. Die ADP/O-Ratio vs. Kontrolle zeigte ausschließlich eine Veränderung in Komplex I unter 179 µM INDO auf 994 % (674 – 1190 %). Zusammenfassend beeinflusste INDO organspezifisch, konzentrationsabhängig und komplexabhängig die mitochondriale Funktion der Colon- und Leberzellen. In Colonzellen wirkte INDO positiv auf die Effizienz der OXPHOS, so dass die Nebenwirkungen auf den Gastrointestinaltrakt vermutlich nicht von einer Dysfunktion der Colonmitochondrien verursacht werden. Im Gegensatz dazu deutet die stark erhöhte ADP/O-Ratio in Leberzellen auf eine spezifische Entkopplung der mitochondrialen Funktion hin, welche die Hepatotoxizität von NSAR erklären könnte.

Abbreviations

°C	degree Celsius
3-MOPS	3-(N-morpholino) propane sulfonic acid
ADP	adenosine diphosphate
ADP/O ratio	amount of ADP to oxygen consumed
α	asymptotic significance
ATP	adenosine triphosphate
BSA	bovine serum albumin
COX	cyclooxygenase
DMSO	dimethyl sulfoxide
EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt
ETS	electron transport system
FAD	flavin adenine dinucleotide
FADH2	reduced FAD
g	gram
INDO	indomethacin
IQR	interquartile range
IU	international units
kg	kilogram
l	liter
M	mole/l
m	milli
mg	milligram
min	minute
ml	milliliter
mM	millimole/l
mm^2	square millimeter
n	number
NAD	nicotinamide adenine dinucleotide
NAD(P)H	nicotinamide adenine dinucleotide phosphate
NAD+	oxidized NAD
NADH	reduced NAD
nm	nanometer
nmol	nanomole
NO	nitric oxide
NSAIDs	non-steroidal anti-inflammatory drugs
OXPHOS	oxidative phosphorylation
pA	picoampere
p.a.	*pro analysi*

RCI	respiratory control index
rpm	rounds per minute
SD	standard deviation
V	volume
vol%	volume percentage
vs.	versus
µ	micro
µM	micromole/l

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1 Introduction

Non-steroidal anti-inflammatory drugs (NSAIDs) are used in a versatile way in many medical fields and for various indications. In general, all NSAIDs share the properties of being analgesic, anti-inflammatory, and anti-pyretic drugs with a wide field of therapeutic effects. NSAIDs are heavily used for the treatment of osteo arthritis, rheumatoid arthritis and pain relief (1) but also play a lifesaving role in pediatrics for the treatment of hemodynamically significant *Patent Ductus Arteriosus* in preterm infants (2). NSAIDs are generally considered to be safe, but it is also known that they show a variety of side effects, for example in 2014 in the United Kingdom 10 % of all hospital admissions of older people have been due to adverse drug reactions of which 30 % have been due to NSAIDs (3).

1.1 NSAIDs – Mode of action

Non-selective NSAIDs inhibit the biosynthesis of prostaglandin through the non-selective blockade of the binding of arachidonic-acid to the enzyme cyclooxygenase (COX) -1 and COX-2. COX-1 is expressed in almost all cells of the human body and regulates homeostasis, for example, it regulates platelet aggregation, normal renal function, protection and mucus production in the gastrointestinal tract. COX-2 is synthesized at the presence of tissue injury in response to cytokines mediating inflammatory pain and fever. COX-2 increases in inflammatory processes leading to an increased pro-inflammatory prostaglandin synthesis (1, 4).

1.2 Side effects of NSAIDs on the human body

It is generally accepted that NSAIDs have potentially toxic side effects on the upper gastrointestinal system and the cardiovascular system, which can be explained by the action of COX-1 inhibition (5). Additionally there is evidence that NSAIDs can induce liver damage (1) as well as small and lower gastrointestinal tract damage which leads to an increased risk for gastrointestinal bleedings (5, 6).

The latter one can be partially explained by an altered mucus and bicarbonate secretion, reduced mucosal blood flow and impaired platelet aggregation due to COX-1 inhibition, making the mucosae more vulnerable to stressors like stomach acid and refluxing bile and as well as by COX-2 inhibition leading to increased leukocyte activation and reduced

angiogenesis impairing healing (7, 8). Further down the intestinal tract injury to the epithelial layer cannot solely be explained by reduced mucus production and low pH, which even increases distal from the stomach and duodenum (5). Next to the systemic effects, there is evidence for NSAIDs exerting injury to the stomach and smaller intestine due to their topical toxicity in addition to COX inhibition (9). These topical effects are mainly thought to occur due to an uncoupled mitochondrial function leading to a lack of adenosine triphosphate (ATP) and consecutive energy shortage to maintain homeostasis (9). However topical pathologic effects are noticeable, the systemic effects seem to overrule as gastric ulceration also occurs when NSAIDs are administered intravenously or as prodrug being activated by the liver metabolism (8). To date, lower GI complications are accounting up to 40 % of the NSAID reported side effects and especially studies on the pathomechanisms of NSAIDs associated to the colon with special regard to mitochondrial function under NSAID therapy are lacking (10). This warrants a call for further research on the mechanisms of the pathogenesis of NSAID associated lower GI complications to guide the prevention and treatment thereof (10, 11).

As mentioned earlier NSAIDs may cause adverse effects on liver function. As much as 10 % of reported drug induced hepatotoxicity is attributed to NSAID treatment (7). Hepatotoxicity caused by the use of NSAIDs ranges from nonsuspicious elevation of liver enzymes like serum aminotransferase to fulminant liver failure and resultant death (12). From literature, it is known that the effects of NSAID induced hepatocyte damage is not due to treatment time, but dose-dependent. Cellular injury and stress is thought to be the result of a bio-energic crisis caused by an uncoupled mitochondrial function, but overall the mechanisms of altered liver function in humans are poorly understood (12, 13).

1.3 Model to explore adverse effects of NSAIDs on colon and liver

The study was set-up to look into the potential adverse effects of NSAIDs on colon and liver *in vitro* on mitochondrial level to find possible explanations for pathomechanisms in colon and liver tissue under the influence of NSAIDs. Animal studies on the mechanism of action of NSAIDs have mainly been conducted on rodents and have been used to find explanatory models of the mode of action of NSAIDs in different organs. Well established models to examine the mitochondrial function of colon and liver in healthy rats have been published and are used for this work to make transfer of study results easier (14-17).

Indomethacin (INDO) was chosen as reference NSAID for this study. INDO is one of the longest known NSAIDs marketed in 1964 (18). INDO shows great potential in combining both anti-inflammatory and analgesic activity (18). Looking at the potency of uncoupling mitochondria *in vitro* INDO belongs to the most cytotoxic NSAIDs together with Flurbiprofen and Ibuprofen (9).

1.4 Effects of INDO on rat organs

1.4.1 Small intestine and Colon

There is ambiguity about the impact of INDO on small intestinal mitochondria. To date, no data is available on the pathological changes with regard to mitochondrial function in the colon either from humans or rodents.

Somasundaram *et al.* (19) looked at the histological changes in the small intestines at 6 hours and focal ulcerative erosions at 20 hours after rats have ingested 5, 20 or 30 mg/kg INDO by gastric gavage. Electron microscopy has revealed mitochondrial changes under the topical influence of INDO indicative for uncoupling of the oxidative phosphorylation (OXPHOS) or inhibition of electron transport system (ETS) as early as one hour after gastric gavage. In their study liver mitochondria treated with INDO were used as an indirect marker to study the effects of INDO on mitochondrial respiration. They assumed that in general there are no differences in the properties of mitochondria regarding the reactions to stimulants or inhibitors of the respiratory chain. From uncoupled OXPHOS and inhibited ETS in liver mitochondria by INDO, it was concluded that the findings from electron microscopy in the small intestines are corresponding to uncoupled mitochondria.

Another study by Basivireddy *et al.* (5) demonstrated a decreased oxygen uptake and a reduced respiratory control index (RCI) compared to the control group, consistent with mitochondrial damage in isolated small intestine mitochondria from rats that have 1 hour for male and 2 hours for female rats prior to sacrifice orally ingested INDO (40 mg/kg). Additionally, viability counts done by trypan blue exclusion on the small intestine from the same rat cohort mentioned before revealed a lower number of viable villus cells in the INDO treated group compared to the control (non-treated) group.

A third research group by Jacob *et al.* (20) looked at the mitochondrial function of INDO on jejunal tissue after oral administration or intrajejunal administration of 20 mg/kg

INDO and *in vitro* incubation in 2.5 mM INDO with a wash out period in buffer solution. They found that *in vivo* mitochondrial function was hampered but the energy charge was maintained and *in vitro* effects of indomethacin are not detrimental but reversible.

Data concerning the effects of INDO on colon mitochondria are lacking completely even though there is evidence that NSAIDs have harmful effects on the lower gastrointestinal tract in humans and a quest for research on the pathomechanism was called from various authors and institutions (6, 10, 11, 13).

1.4.2 **Liver**

There is uncertainty about which dose uncouples or inhibits mitochondrial respiration in hepatic mitochondria. Data about hepatic rat mitochondria suggest that INDO at low doses has a rather stimulating effect and in higher doses uncouples the OXPHOS from the ETS.

Somasundaram *et al.* assumed for their study on the damaging effects of INDO on rat small intestine that there are no major different reactions to uncoupling and inhibiting agents on mitochondria from different organs. In this study it was found that INDO uncoupled the ETS from the OXPHOS in concentrations of < 200 µM, and reducing the oxygen consumption in higher concentrations > 300 µM (19).

Mahmud *et al.* (21) presented different results when investigating the effects of INDO on liver homogenates. Stimulating effects on mitochondrial oxygen consumption occurred between 0,02 and 2,7 µM INDO/mg protein (equals 0,006 – 8,1 µM in the study design of this work) and inhibiting effects became evident above 2,7 µM/mg protein (equals 8,1 µM in study design of this work) when uncoupling was already apparent.

1.5 **Aims of thesis**

Taken together, this study was designed to explore the mitochondrial function of colon and liver tissue under different doses of INDO *in vitro* for a possible explanation of the pathomechanisms behind the adverse effects of INDO seen in colon and liver.

The following research question was defined:
What are the effects of INDO on colonic and hepatic mitochondria and are these effects organ specific and concentration dependent?

2 Materials & Methods

NOTE: Parts of this section have been published in the Master thesis (14) from the author of this work and are only mildly adapted to the purposes of this study.

2.1 Preliminary experiments

The organ preparation and the basic measurement set-up were used as earlier described (14, 16).

2.2 Chemicals, reagents and laboratory equipment

A complete list of all chemicals, reagents, laboratory equipment and their respective provider can be found in the appendix. Some mentions of chemicals, reagents and laboratory equipment are in the text for better understanding and are listed again in the appendix. Buffer solutions and their constituents can be found in the text for better reading.

2.2.1 Preparation of INDO dilutions

INDO was diluted in Dimethyl sulfoxide (DMSO). DMSO was chosen as diluent as it is a standard drug carrier in mitochondrial research and shows only minor toxicity on mitochondrial respiration when used in low concentrations (16, 21, 22). The final concentration in the respiration chamber was calculated to be less than 2 % in all experiments. Aliquots of INDO dilutions have been produced on stock and were refrigerated at -20°C. The INDO dilution was later pipetted to the tissue homogenates to yield the following concentrations in the respiration chamber:

Colon homogenate: 0.36, 1, 30, 179, 300, 1000, 3000 µM

Liver homogenate: 0.36, 1, 3, 10, 30, 100, 179 µM

2.2.2 Preparation of respiratory substrates

Chemicals prepared for respiratory research and proof for mitochondrial integrity have been produced on stock, aliquoted and refrigerated at -20°C until use. A list of these chemicals, its diluents and concentrations are presented in table 1.

Substrate	Diluent	Concentration in respiration chamber	
		Colon	Liver
ADP	H_2O	50 µM	250 µM
Cytochrome C	H_2O	2.5 µM	2.5 µM
Glutamate	H_2O	2.5 mM	2.5 mM
Malate	H_2O	2.5 mM	2.5 mM
Oligomycin	DMSO	0.05 µM	0.05 µM
Rotenone	DMSO	0.5 µM	0.5 µM
Succinate	H_2O	5 mM	10 mM

Table 1: Respiratory substrates

Abbreviations: ADP – adenosine diphosphate, H_2O – water; Units: µM – micromole/liter, mM – millimole/liter

2.3 Animals

The study was approved by the Animal Ethics Committee of the University of Düsseldorf. A permit to work with animal-tissue for research projects or educational purposes has been issued by the University of Düsseldorf (Az ZETT: O27/12). For this study there was no need for a certificate to handle living animals as it was conducted only with animal tissue from dead animals. The rats and the harvested tissues have been handled in agreement to the Guide for the Care and Use of Laboratory Animals (23).

For this study only male *Wistar* rats have been used to exclude hormonal irregularities. Rats from Janvier Breeds in France have been ordered from the animal institution of the University of Düsseldorf in cooperation with another research group from the University of Düsseldorf who took over the animal handling. In total, organs from 48 rats were included in this study.

2.3.1 Animal handling

The rats were bred at an artificial 12-h day/night cycle at constant temperature and defined humidity, having free access to standard feed and tap-water. The rats used were approximately 3 months old and had an average bodyweight of 240-280 g. Rats received intraperitoneal anesthesia with 90 mg/kg sodium pentobarbital mixed with 1000 IU Heparin and 1.8 ml 0.9% sodium chloride. After 4 minutes the rats were weighed and at minute 5, under deep areflexia, they were sacrificed by decapitation.

2.4 Organ preparation

2.4.1 Organ isolation

Within one minute after decapitation the beating heart was obtained by thoracotomy from another research group. Within the second minute after decapitation, the right lobe of the liver and the colon were isolated by full laparotomy.

2.4.2 Preparation of colon homogenates

The freshly harvested colon was squeezed out to remove the feces and placed into 10 ml ice-cold isolation buffer (Table 2). The wet weight of the colon was then measured. After this, the colon was longitudinally cut open, gently dried and cleaned with a gauze pad to remove remaining feces and mucus. Quickly after this procedure the colon was trypsinized with a weight adjusted volume (V = wet weight (g) x 2 ml) of 0.05 % trypsin and incubated for 5 minutes on ice to loosen the intracellular bonds. Next, the colon was placed in ice-cold isolation buffer containing 2 % bovine serum albumin (BSA) (Table 3) (V = wet weight (g) x 4 ml) and protease inhibitors cOmplete^{TM} (V = wet weight (g) x 40 µl), cut into 2-3 mm^2 pieces and homogenized with a teflon homogenizer (Potter-Elvehjem, 2000 rounds per minute (rpm), 5 strokes) in an ice-jacketed homogenization cylinder. Following this procedure, the homogenate was strained through a gauze swab (ES-Kompressen, 5x5cm, 17 threads, 12 layers, Paul Hartmann AG) into a 50 ml falcon and put-on ice until further use.

2.4.3 Preparation of liver homogenates

Straight after full laparotomy the right lobe of the liver was harvested and put in 10 ml ice-cold isolation buffer (Table 2). 1.1 g of liver tissue was weighed and minced into 2-3 mm^2 pieces with scissors. Carefully the surplus of isolation buffer was decanted to wash out erythrocytes and fresh isolation buffer was added to get a dilution of 1:10 ml. The liver suspension then was homogenized with a teflon homogenizer (Potter-Elvehjem, 2000 rpm, 5 strokes), while the homogenization cylinder was ice jacketed. Next, the homogenate was strained through a gauze swab into a 50 ml falcon and contained on ice until further use.

Chemical	Concentration
Mannitol	200 mM
Sucrose	50 mM
Potassium dihydrogen phosphate	5 mM
3-MOPS	5 mM
EGTA	1 mM
BSA	0.1 %
Potassium hydroxide (4 M)	To adjust pH to 7.15
Diluent	Pure water

Table 2: Isolation buffer for explantation of colon and liver and for production of liver homogenate
Abbreviations: 3-MOPS – 3-(N-morpholino) propane sulfonic acid, BSA – bovine serum albumin, EGTA – ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt; Units: mM – millimole/liter, M – mole/liter

Chemical	Concentration
Mannitol	200 mM
Sucrose	50 mM
Potassium dihydrogen phosphate	5 mM
3-MOPS	5 mM
EGTA	1 mM
BSA	2 %
Potassium hydroxide (4 M)	To adjust pH to 7.15
Diluent	Pure water

Table 3: Isolation buffer for the production of colon homogenate
Abbreviations: 3-MOPS – 3-(N-morpholino) propane sulfonic acid, BSA – bovine serum albumin, EGTA – ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt; Units: mM – millimole/liter, M – mole/liter

2.5 Determination of protein concentration in colon and liver homogenates

2.5.1 The Lowry method

The protein concentration in both homogenates was determined using an adapted protocol based on the Lowry method (24). This method is based on the Biuret and Folin-Ciocalteu reaction. During the first reaction, the Biuret reaction, purple complexes are formed between the peptide bonds from the dissolved protein and copper(II)-ions in an alkaline solution. The quantity of formed complexes reflects the number of peptide bonds. The second reaction, the Folin-Ciocalteu reaction, is a reduction of copper(II) to copper(I)

through oxidation with aromatized amino acids. Copper(I) then reduces the Folin-Ciocalteu reagent to form molybdenum blue. The absorbance at 750 nm is then measured using a photometer to quantify the blue stain. To quantify the protein concentration of a probe, the extinction of a serial dilution of a probe of a known protein concentration has to be put in relation to the known protein concentration. Using the Lambert-Beer's law, the protein concentration of the probe can be calculated using its extinction.

2.5.2 **Practical implementation**

The standard protein for the serial dilution was BSA. Pure water was used as a dilutive. The concentration of the standard dilutions used for plotting the standard curve are summarized in table 4. The serial dilution was produced freshly every day and stored on ice in 1,5 ml reaction tubes.

Probe #	**1**	**2**	**3**	**4**	**5**	**6**	**7**
BSA concentration [µg/ml]	1000	750	500	250	125	62,6	0

Table 4: Standard BSA concentration in serial dilution for probe protein determination
Abbreviation: BSA – bovine serum albumin; Units: µg/ml – microgram/milliliter, # – number

For protein determination a probe of the single organ tissue homogenate has been diluted 1:100 in pure water. For each organ, three technical replicates were performed. Thereafter, 100 µl from the serial solution respectively the homogenate dilution was mixed with 500 µl of Lowry-Solution-1 (Table 5) and being vortexed for 2 seconds and incubated at room temperature for 10 minutes. After this first incubation period 50 µl Lowry-Solution-2 (Table 5) was added into every reaction tube. All solutions were vortexed for 2 seconds and incubated for another 30 minutes and were vortexed again for 2 seconds. For every probe a double control for the determination of the protein concentration was done. For this purpose, 200 µl per well were pipetted into a 96-well plate. Subsequently the extinction was measured at 750 nm and the protein concentration was calculated (Synergy^{TM}2 Multi-Mode Microplate Reader, software version: Gen 5.1).

Lowry-Solution 1 Mixed from reagent A, B, C Ratio A:B:C = 1000:1:1	
Reagent A	0.1 M Sodium carbonate 0.047 M Sodium hydroxide Solubilized in pure water
Reagent B	0.095 M Potassium sodium tartrate tetrahydrate Solubilized in pure water
Reagent C	0.08 M Copper (II) sulfate pentahydrate Solubilized in pure water
Lowry-Solution 2 Mixed from reagent A, B Ratio A:B = 1:1	
Reagent A	Folin & Ciocalteu phenol reagent, Sigma Aldrich
Reagent B	Pure water

Table 5: Lowry-Solution 1 & 2: Proportions and composition

Units: M – mole/liter

2.6 Determination of mitochondrial function

2.6.1 Mitochondrial respiration

Mitochondrial respiration takes place in the inner mitochondrial membrane through five multimeric protein complexes. Complex I oxidizes NADH to NAD^+ and Complex II reduces FADH2 to FAD, donating electrons which are transported along the complexes to form water with molecular oxygen at complex IV. This electron transport utilizes two inter membrane electron carriers: ubiquinone (coenzyme Q10) between complex II and III and cytochrome C between complex III and IV. Simultaneously, complex I, III, and IV are pumping protons across the inner membrane, creating a gradient which drives hydrogen back through complex V, the ATP-synthase, hydrolyzing adenosine diphosphate (ADP) to ATP (25). The synthesis of ATP by mitochondria is called OXPHOS.

2.6.2 Mitochondrial respiration rates

In mitochondrial state 2 respiration there is an abundance of substrate for either complex I or II. Complex I is triggered by glutamate and malate. Glutamate is oxidized by the enzyme glutamate dehydrogenase to α-ketoglutarate, a reaction leading to the reduction

of NAD^+ to NADH being an electron donor for complex I. Simultaneously, malate is being oxidized by the enzyme malate dehydrogenase to oxaloacetate by the reduction of NAD+ to NADH, which as well serves as an electron donor to complex I. Succinate acts as substrate for complex II. Succinate is oxidized by succinate dehydrogenase, which is coupled with the reduction of FAD to FADH2 serving as an electron donor for complex II. To be able to differentiate between the function of complex I and II *in vitro*, complex I can be blocked by adding rotenone prior to the addition of succinate. Rotenone is preventing the back flow of electrons from complex II to complex I. State 3 respiration is measured under the presence of substrate and ADP. ADP serves as the strongest activator of the respiratory chain (26), leading to a sudden increase of oxygen consumption. As soon as ADP is used up, a steady state respiration is reached which is called state 4 respiration. A limiting factor for state 3 respiration, beside the concentration of ADP, is the depletable oxygen concentration in the surrounding medium. To define a measure of the coupling between the ETS and the OXPHOS the RCI is calculated by dividing state 3 by state 2. A second measure to reflect the efficiency of OXPHOS is the ADP/O ratio being calculated from the amount of ADP added and the O_2-consuption measured during state 3 (Fig. 1).

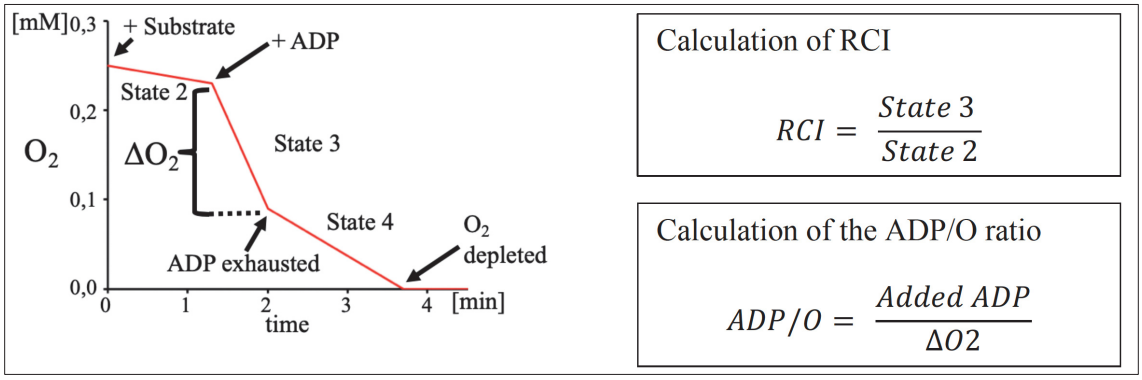

Fig. 1: Schematic depiction of respiratory rates (left). Calculation of RCI and ADP/O ratio (right). State 2 – Substrate dependent respiration, State 3 - ADP dependent respiration, State 4 – respiration after ADP depletion, ADP/O ratio – measure of efficiency of oxidative phosphorylation, RCI – measure of coupling between the electron transport system and the oxidative phosphorylation; Abbreviations: ADP – adenosine diphosphate, O – oxygen; RCI – respiratory control index, Δ – difference; Units: mM – millimole/liter, min – minute

2.6.3 **Measurement of mitochondrial respiratory rates**

For the measurement of the mitochondrial function, the oxygen uptake rate was polarographically measured at 30 °C using a Clark type electrode (model 782,

Strathkelvin instruments, Glasgow, Scotland). The thermostatic water-jacketed respiration chamber had a volume of 0.5 ml, air-locked by a plastic plunger with a capillary opening for micro syringes and constant magnetic stirring. The electrode was calibrated daily, according to the manufacturer's specifications, using pure water as a high control and a near saturated sodium sulfite solution as low control. The high values were between 200 and 600 picoampere (pA) and the low values between 0 and 50 pA. The oxygen solubility was assumed to be 223 µmol O_2/l at 30 °C. The change in oxygen concentration in the respiration chamber was plotted graphically and expressed in nmol/min/(mg protein).

2.6.4 **Measurement protocol**

Aliquots of tissue homogenates have been resuspended in respiration buffer (Table 6) prior to the single experiment, to yield a protein concentration of 4 mg/ml for liver and 6 mg/ml for colon.

Chemical	**Concentration**
Potassium chloride	130 mM
Potassium dihydrogen phosphate	5 mM
3-MOPS	20 mM
EGTA	2.5 mM
Sodium Phosphate	1 µM
BSA	2 %[1]; 0.1 %[2]
Potassium hydroxide (4 M)	To adjust pH to 7.4
Diluent	Pure water

Table 6: Respiration buffer for colon[1] and liver[2] homogenates

Abbreviations: 3-MOPS – 3-(N-morpholino) propane sulfonic acid, BSA – bovine serum albumin, EGTA – ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid tetrasodium salt; Units: µM – micromole/liter, mM – millimole/liter, M – mole/liter

2.6.4.1 **Mitochondrial integrity**

During preparation of the tissue homogenates, mitochondrial membrane damage can occur. Mitochondrial integrity can be checked by adding cytochrome C, which serves as a coenzyme for complex IV. When cytochrome C is provided and the oxygen consumption is stable, it can be assumed that the outer membrane is integer (27). To check integrity of the inner membrane oligomycin as ATP-synthase inhibitor is applied. When

the inner membrane is integer, the non-phosphorylating state stays analogous to state 2 (28, 29).

A quality control for both homogenate resuspensions from colon and liver respectively have been checked for mitochondrial membrane integrity with an abundance of substrate and by adding 2.5 µM cytochrome C and 0.05 µg/ml oligomycin. The experiment was only continued, when there was no increase in flux after addition of cytochrome C and the non-phosphorylating state was analogous to state 2 when oligomycin was added.

2.6.4.2 **Mitochondrial respiration under the presence INDO**

The resuspended homogenates were incubated in 1.5 ml reaction tubes for 3 minutes with either the same volume DMSO (control) or with a given concentration of INDO at room temperature kept at 21 °C (Fig. 2).

Action	Mixing of homogenate with respiration buffer	Adding DMSO or INDO	Transfer to respirometer
Time [minutes]	0	3	6

Fig. 2: Timeline of incubation with DMSO or INDO for colon and liver homogenates respectively
Depiction of time of adding respiration buffer and DMSO (control) or INDO to homogenate (colon, liver) and time of incubation with either substance; Abbreviations: DMSO – dimethyl sulfoxide, INDO – indomethacin

This protocol was adapted based on prior research by Herminghaus & Buitenhuis *et al.* (16). Preliminary experiments have shown that, at a time period of 3 minutes of incubation, INDO exhibits consistent results on the parameters measured than at shorter periods (preliminary data not shown). After the incubation period, the suspensions were transferred into the respiration chamber. After an equilibration period of 1-minute mitochondrial state 2 respiration was measured, either in the presence of glutamate and malate (either 2.5 mmol) or in the presence of succinate and rotenone (succinate: 5 mmol for colon, 10 mmol for liver; either 0.5 µmol rotenone). Rotenone was added at timepoint 0. At minute 2 ADP (50 µmol for colon; 250 µmol for liver) was added. In total 6 minutes were recorded for experiments with colon homogenates and 4 minutes were recorded for experiments with liver homogenates (Fig. 3). Three technical replicates were performed for colon and two for liver.

Substrate	Complex I	Glutamate & malate	ADP	
	Complex II	Rotenone	Succinate	
Action		Start	→	→ END
Time [minutes]		0	1	2 4^1 / 6^2

Fig. 3: Timeline of respirometry ([1]liver, [2]colon)

Depiction of time of addition of rotenone (inhibition of complex I), substrates (glutamate & malate or succinate) and ADP and the sequence of respirometry; Abbreviation: ADP – adenosine diphosphate

2.7 Statistics

2.7.1 Power analysis

A power analysis was performed using SigmaStat (Version 4.0, Licensed for the University of Düsseldorf) for sample size calculation. Based on a standard deviation of 0.15 % and difference in means of 35 %, setting power to > 0.85 and an α-error of 0.05, it was calculated that the sample size should be n = 6 animals per group. The assumptions of difference in means and standard deviation were made from earlier experiments in the department of anesthesiology at the University of Düsseldorf.

2.7.2 Data acquisition

Three technical replicates for colon and two for liver were performed. The arithmetical mean for every measure of the total of technical replicates per experiment was formed. The data output was saved in excel (Microsoft Excel 97-2004, Workbook, Version 11.0, University of Düsseldorf). RCI and ADP/O were calculated as percentage of the control, which is the measurement of mitochondrial respiration after incubation with only DMSO at room temperature.

2.7.3 Statistical analysis

The accumulated data output was converted in percentages of the control value (set at 100%) using excel. These data were then transferred to PRISM-statistics (GraphPad Prism, Version 8.0, GraphPad Software, Inc, La Jolla, USA). Testing for normality with the Kolmogorov-Smirnov-Test showed a non-parametrically distributed data set. These non-parametric data were analyzed utilizing the Kruskal-Wallis test of variance. To check if specific medians were significantly different from each other a Dunn's correction was

performed. Differences in medians with $\alpha < 0.05$ were considered significant. Median, inter quartile range (IQR), minimum and maximum were determined. Only median and IQR are reported in text in the following annotation: median, IQR (lower 25th percentile to upper 75th percentile). Data were graphically presented as Box-Whisker-Plots using GraphPad Prism (Version 8.0, GraphPad Software, Inc, La Jolla, USA) for state 3, the ADP/ratio and the RCI.

3 Results

The effects of INDO on mitochondrial OXPHOS, reflected by the efficiency (ADP/O) and the coupling between the ETS and OXPHOS reflected by the RCI from complex I and complex II are presented compared to the control (DMSO) set at 100 %. Only results with a statistical significance of $\alpha < 0.05$ are presented for state 3 respiration, the RCI and the ADP/O ratio.

3.1 Colon

The results of the experiments with colonic mitochondria are displayed in Fig. 4.

3.1.1 Complex I

While state 3 was reduced at a concentration of 1000 and 3000 µM INDO (control: 100 %; INDO: 1000 µM: 67 % (63 - 74 %); 3000 µM: 51 % (41 - 58 %)), no statistically significant changes could be demonstrated for the RCI compared to control. An increase in efficiency of oxygen utilization reflected by an increased ADP/O was seen at 1000 and 3000 µM INDO (control: 100 %; INDO: 1000 µM: 159 % (133 - 166 %); 3000 µM: 183 % (167 – 349 %)).

3.1.2 Complex II

State 3 was reduced at 179 and 3000 µM INDO (control: 100 %; INDO: 179 µM: 75 % (68 – 84 %); 3000 µM: 63 % (48 – 69 %)). The RCI remained unaltered compared to control in complex II under the influence of INDO. An increase efficiency of oxygen utilization could be demonstrated by an increased ADP/O ratio for both 179 and 3000 µM INDO (control: 100 %; INDO: 179 µM: 142 % (113 – 150 %); 3000 µM: 175 % (140 - 238 %)).

3.2 **Liver**

The results of the experiments with hepatic mitochondria are displayed in Fig. 5.

3.2.1 **Complex I**

A decrease of state 3 was revealed at 100 and 179 µM INDO (control: 100 %; INDO: 100 µM: 65 % (58 - 66 %); 179 µM: 41 % (34 - 49 %)). The coupling between the OXPHOS and ETS reflected by the RCI decreased only at 179 µM INDO (control: 100 %; INDO: 179 µM: 41 % (32 - 50 %)). In contrast the ADP/O ratio increased at 179 µM INDO (control: 100 %; INDO: 179 µM: 994 % (674 – 1190 %)).

3.2.2 **Complex II**

State 3 stood unaltered while the RCI showed an altered coupling at 179 µM INDO (control: 100 %; INDO: 179 µM: 73 % (66 - 75 %)). The ADP/O reflected neither a trend nor a significant change of efficiency in oxygen utilization.

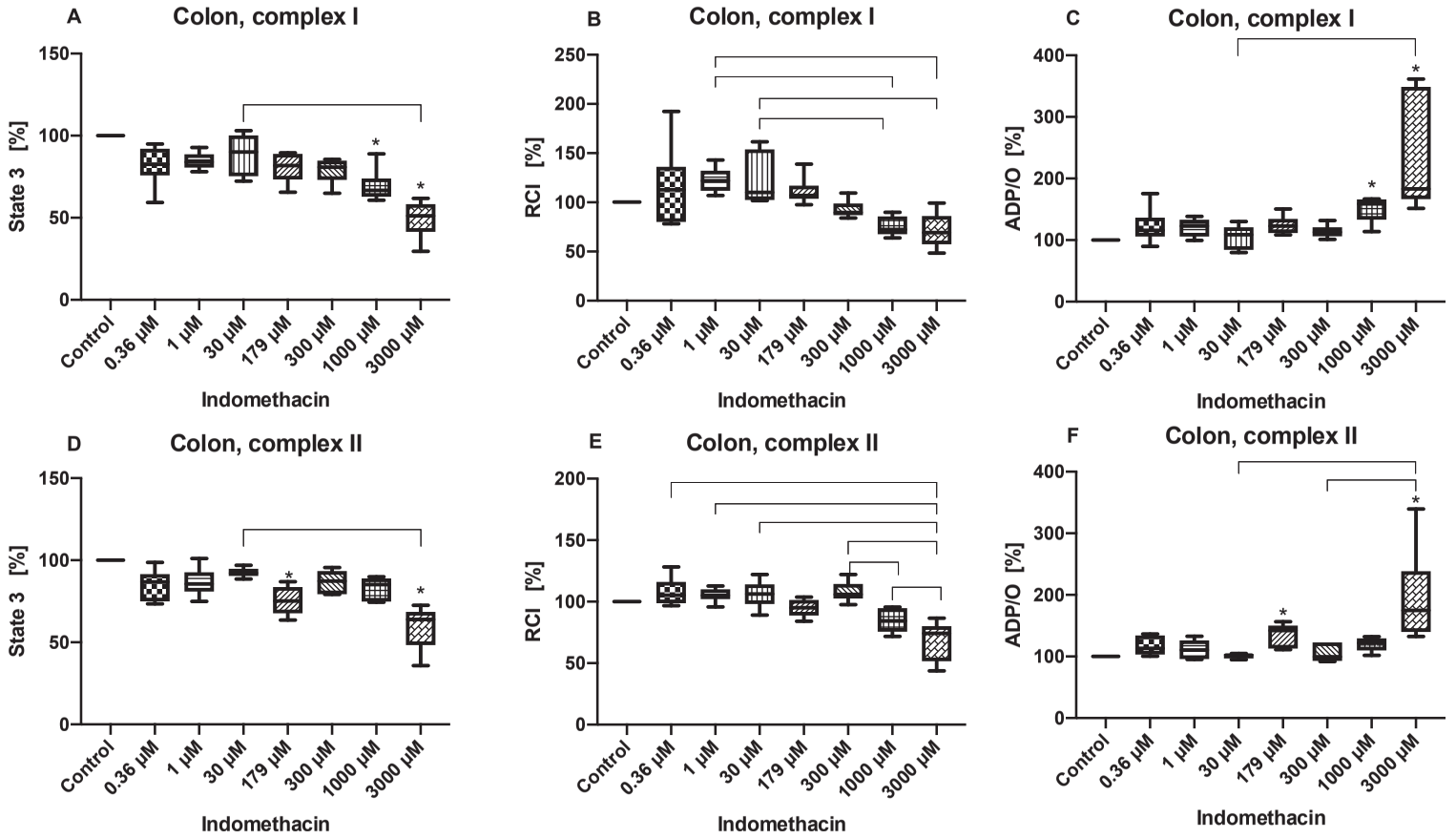

Fig. 4: Influence of INDO in different concentrations (0.36, 1, 30, 179, 300, 1000 and 3000 µM) on mitochondrial function of colonic tissue homogenates

State 3 after stimulation trough complex I (A) and complex II (D), RCI for complex I (B) and complex II (E) and ADP/O ratio for complex I (C) and complex II (F). Data are presented as Box-Whisker-Plots presenting median, interquartile range, minimum and maximum value, n = 6, * $p < 0.05$ vs. control, ⌐¬ $p < 0.05$ between groups; Abbreviations: ADP – adenosine diphosphate, O – oxygen, RCI – respiratory control ratio; Unit: µM – micromole/liter

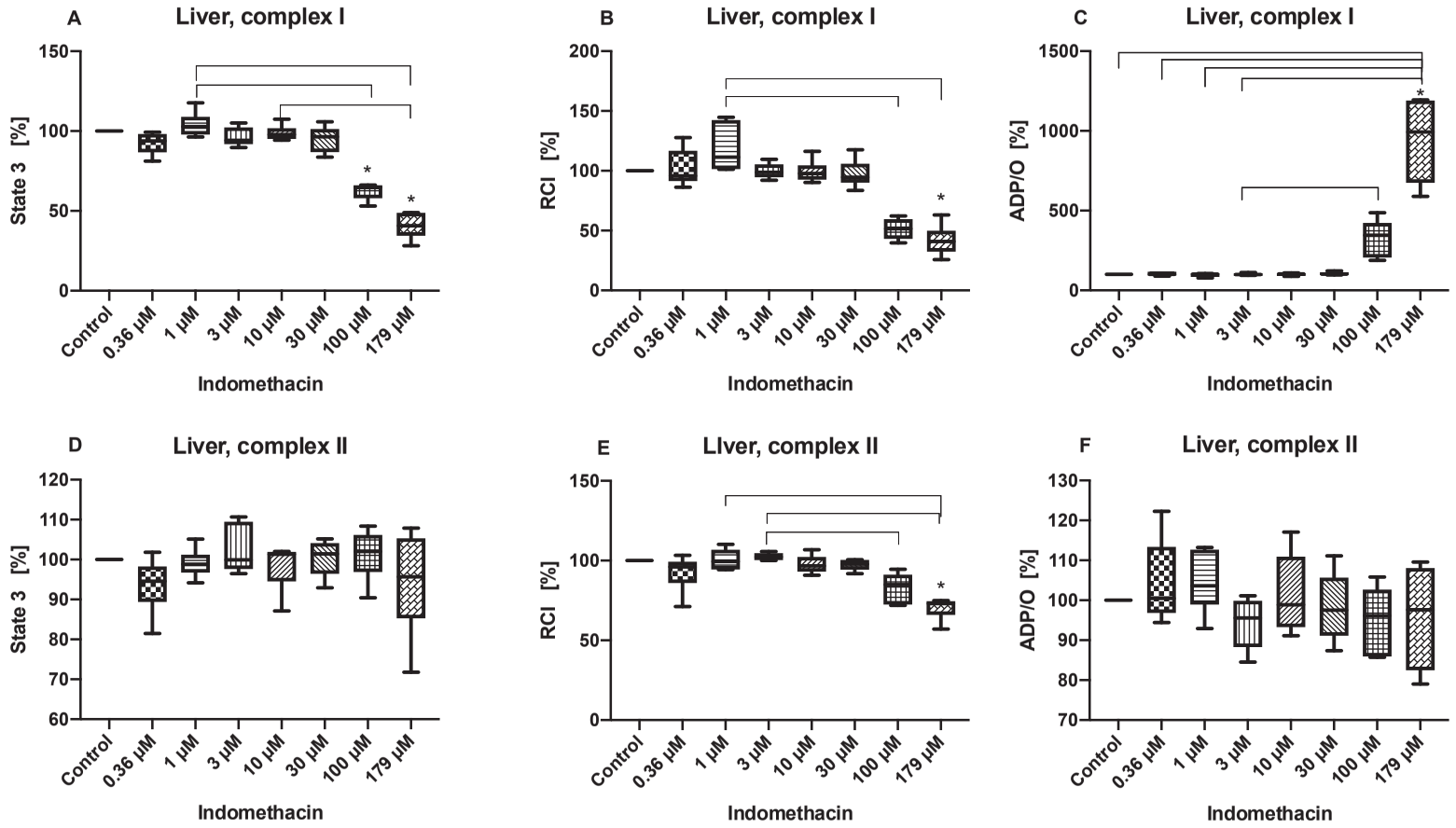

Fig. 5: Influence of INDO in different concentrations (0.36, 1, 3, 10, 30, 100, 179 µM) on mitochondrial function of hepatic tissue homogenates

State 3 after stimulation trough complex I (A) and complex II (D), RCI for complex I (B) and complex II (E) and ADP/O ratio for complex I (C) and complex II (F). Data are presented as Box-Whisker-Plots presenting median, interquartile range, minimum and maximum value, n = 6, * $p < 0.05$ vs. control, ⌐¬ $p < 0.05$ between groups; Abbreviations: ADP – adenosine diphosphate, O – oxygen, RCI – respiratory control ratio; Unit: µM – micromole/liter

4 Discussion

The study was designed to identify the concentration dependent alterations of mitochondrial function of colonic and hepatic mitochondria under the presence of INDO as a possible model of explanation of the pathomechanisms of the adverse effects of NSAIDs seen in colon and liver.

The main results of this study reflecting on the research question mentioned in the introduction: INDO affects colonic and hepatic mitochondria in an organ-specific and concentration-dependent manner. INDO appears to have a positive effect on colonic mitochondria, showing an unaltered RCI whereas the ADP/O ratio increased. On hepatic mitochondria INDO seems to have a rather deteriorating effect. At the highest concentration INDO decreased the RCI for both complexes in hepatic mitochondria. In complex I stimulated respiration the ADP/O ratio was indicative for terminal uncoupling at the highest concentration of INDO.

Taken together, these results do not explain injury to colonic tissue but may contribute to the explantation of hepatotoxicity seen with NSAIDs.

4.1 Colonic mitochondria under the influence of INDO

In colonic mitochondria, INDO reduced dose dependently (1000 and 3000 μM INDO for complex I and 179 and 3000 μM INDO for complex II), ADP-induced state 3 respiration, without altering the RCI compared to control. This is due to an analogue altered state 2 respiration (data not shown) leading to non-statistical changes of the quotient, as the RCI is the quotient of state 3 divided by state 2, which can be interpreted as unchanged coupling between the ETS and OXPHOS. Without changes of the RCI, the efficiency of the OXPHOS has increased dose-dependently in both complexes shown by an increased ADP/O ratio. This is understood as a positive adaptation as the production of ATP is more efficient at less oxygen costs. It has to be recognized that complex II shows no alteration of state 3 respiration at concentrations up to 1000 μM INDO compared to control. To gain further knowledge about the complex specificity, further research into the single complex functions and activities under the influence of INDO is warranted.

The experiments of this work indicate a rather positive effect on colonic mitochondria under the influence of INDO. In contrast small intestinal mitochondria showed a

decreased RCI one up to two hours after orally ingesting INDO. This indicates mitochondrial damage as demonstrated by Basivireddy *et al.* (5). Comparison of this work to the observations from Basivireddy *et al.* has to be done with reservation as the study design differs (*in vitro vs. in vivo*). In this study incubation with INDO took place *in vitro*, whereas Basivireddy *et al.* fed INDO orally. Systemically mediated COX inhibition may play a role as well as topical (luminal) and tissue concentrations of INDO which are not known. Looking into the literature, plasma levels of INDO and calculated levels of hepatic drug concentrations can be found (30), but no data on luminal and duodenal or colonic tissue concentrations are available. Lastly, comparing tissues with different properties and functions with each other is a skewed comparison.

Even Jacob *et al.* (20) presented contradicting results with comparable drug concentrations to this study showing no changes in oxygen uptake no matter if *in vivo* or *ex vivo.* Again, this comparison is skewed, as jejunum and colonic tissues have different properties and their experiments were conducted on full tissue, whereas the experiments in this work were conducted on tissue homogenates.

The results found in this experimental set-up have been unexpected because the formation of ulceration in the colon may not be explained by an altered mitochondrial function.

4.2 Hepatic mitochondria under the influence of INDO

The results concerning the effects of INDO on hepatic mitochondrial function show new findings. It has been demonstrated that INDO not only shows a dose-dependent effect on respiratory rates but also a complex specific effect on hepatic mitochondria.

Dose dependent effects for complex I show at the highest concentration of 179 µM INDO a significant decrease of state 3 respiration without a change of state 2 and state 4 respiration (data not shown). The decreased state 3 respiration leads to a significantly decreased RCI. As both state 2 and state 4 are unaltered, it can be considered that respiration is dampened. This can be underlined by a massively increased ADP/O ratio due to a significantly decreased ΔO (data not shown). In other words, only a little oxygen is consumed during ADP-dependent respiration. From this it can be concluded that complex I dependent respiration is discoupled by extrinsic toxic stress (31). Discoupling seems only to be present under complex I mediated respiration. Complex II showed a mild but significant alteration of the RCI at a concentration of 179 µM INDO, reflecting

an altered coupling between ETS and OXPHOS. This can be explained by a slight but non-significant increase in state 2 respiration compared to control (data not shown) decreasing the quotient (RCI). With an evenly risen state 4 respiration (data not shown) and a non-altered ADP/O ratio, it can be concluded that vectorial transmembrane proton flux from complex II downstream of the ETS is only mildly affected by high concentrations of INDO and that these changes in proton flux have no measurable effect on the efficiency of OXPHOS (31). It can be hypothesized that under abundance of succinate energy balance of liver cells is not altered by bypassing complex I.

These aforementioned findings are contradictive with the proposed model in which NSAID translocate into the inner mitochondrial membrane and create ionophores alike complex V (ATP-synthase), bypassing complex V, allowing hydrogen ions to enter the inner mitochondrial matrix without the production of ATP, consequently ATP is depleted over time (9). This hypothesis would be supported if state 2 and state 4 respiration would be higher than the control. With the findings from this work this model has to be adapted, as it seems that complex I is altered as no electron gradient can be established looking at the low ΔO. Oxygen is reduced through complex IV reducing oxygen to water at the cost of 4 electrons from the inner membrane space and pumping net 2 protons (H^+) into the inter membrane space (32). From the data of this experimental set-up, it cannot be derived that no ATP is produced, but it can be manifested that no electron gradient has been built up from complex I. Thus complex I must be blocked or altered in its function. This can be underlined with non-statistically significant changes of state 2 (compared to control) (data not shown), state 4 (data not shown) and state 3 respiration under the presence of complex II substrate succinate. Up to now, it was reported that INDO inhibited ETS in complex I, II and III in a concentration dependent manner (19). This specificity for altering complex I function of hepatic mitochondria is also reported in a study from Sandoval-Acuna *et al. (33),* who found a specificity of complex I inhibition in rat duodenal epithelial cells and Caco-2 cells at concentrations of already 5 μM INDO with only mild affection of complex II activity. Sandoval-Acuna *et al.* measured complex I activity spectrographically at a wavelength specific for NADH, measuring the reduction of NADH to quantify complex I specific activity. Their experiment setup is mutually different from the work presented but underlines the findings from this work and therefore the results should be carefully appreciated.

In addition to the complex specificity, the findings from Somasundaram *et al.* (19) could also not be reproduced. In their study INDO uncoupled the ETS from the OXPHOS at concentrations < 200 µM INDO and reduced the oxygen consumption in concentrations of > 200 µM INDO for complex I, II plus III. This study could already reveal a significant discoupling and significantly decreased oxygen consumption at 179 µM INDO, but only for complex I in hepatic mitochondria. Consistently, the findings from Mahmud *et al.* (21) could not be reproduced where even lower INDO concentrations (8,1 µM (value adapted to this study setup)) inhibited mitochondrial respiration under the presence of succinate (complex II mediated respiration).

4.3 Methodological discussion

The method for preparation of tissue homogenates and respiratory rate measurements and calculation was based on previous established experiment set-ups (14-17). The experimental measurement protocol was based on the setting used in a previous publication (16). The concentrations of INDO for liver were chosen corresponding to earlier *in vitro* experiments (19, 21) and based on preliminary experiments for colon. Incremental concentrations were implemented to be able to make a statement about a possible concentration dependency of colon and liver functions to INDO. Different maximal INDO concentrations were applied to colonic and hepatic tissue homogenates. Discoupling of the ETS from the OXPHOS from complex I was observed as early as at 179 µM INDO in hepatic mitochondria. At this concentration colonic mitochondria did hardly show any alteration of mitochondrial respiration. Therefore, colonic mitochondria were treated with higher concentrations of INDO.

The INDO concentrations used are highly clinically relevant and are within and above the clinical apparent plasma levels. In humans after an administration of an oral dose between 75 and 200 mg INDO per day the plasma concentration is about 0.3 µM (34) and in rats after an administration of 10 mg/kg per day does reach 140 µM (35).

This experiment was conducted on full tissue homogenates *in vitro,* therefore no statement can be made on the mitochondrial function of a single cell line nor a statement can be made about the *in vivo* mitochondrial function from colonic and hepatic mitochondria. Especially colonic homogenates consist of different cell lines (epithelial cells, smooth muscle cells, adipocytes and others) whereas hepatic homogenates are

mainly composed of hepato- and adipocytes. Therefore, mitochondrial function of a single cell line cannot be examined and especially oxygen consumption of colonic homogenates may be blended by a dominating cell line.

Measurements of mitochondrial function were performed at 30°C which is not physiological but a methodological standard in mitochondrial research (15-17, 19, 21). As drug kinetics, pH and mitochondrial function can be altered by temperature, these data can only be transferred to living organisms with reservation. Respiratory rates may not only be inhibited by the lack of substrate, but also by the lack of oxygen, because the respiratory chamber is airlocked, for reasons of standardization and calculation of oxygen content and consumption in a given volume. In experiments using a Clark-type electrode the natural drift interferes with the results. For this experimental setting, no correction was done as a drift of < 0.5 % per 12 hours is neglectable in an experiment not lasting more than 6 minutes ($7*10^{-4}$ %/min).

The transfer of experimental results from *in vitro* mitochondrial respiration of rat mitochondria to the human model is impossible. Not only because rat mitochondria may exhibit different properties compared to human mitochondria, but also because the experimental artificial conditions may influence the mitochondrial function. Results can rather be seen as an indicator to which direction mitochondrial function would go under the presence of INDO.

5 Conclusion

This study is the first to describe the effects of INDO on colonic mitochondria. INDO shows organ- and complex-specific effects in a dose-dependent manner on mitochondrial respiration *in vitro*. In colonic mitochondria INDO shows a rather positive effect on mitochondrial respiration, while in hepatic mitochondria INDO discouples the OXPHOS from the ETS when stimulated through complex I. INDO did not show deleterious properties on colonic mitochondria in the used concentrations, therefore, this work does not give a hint towards the possible pathomechanisms of lower GI complications dreaded due to NSAID treatment. More research is warranted to find out about the pathomechanisms to possibly prevent or reduce the adverse events on the lower GI-tract. The principle of hepatotoxicity, as earlier explained in the introduction, may be

underlined by the results of this study. Nevertheless, a transfer of *in vitro* animal experimental results to human medicine has to be gauged with reservation.

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7 Appendix

7.1 Material and instruments for animal handling

0.9 % NaCl	Isotonische Kochsalzlösung, Fresenius Kabi, Germany
Cannula	Sterican® Standardkanüle Gr. 2, G21 x 1 1/2 Zoll 0,80 x 40 mm grün, B. Braun Melsungen AG, Germany
Disposable syringe	Injekt® 5 ml, B. Braun Melsungen AG, Melsungen, Germany
Heparin	Heparin Natrium, B. Braun Melsungen AG, Germany
Scale	EMB 2200-0, KERN & Sohn GmbH, Germany
Sodium pentobarbital	Nacoren, Boehringer Ingelheim, Germany

7.2 Material and instruments for laboratory work

Crush ice machine	AF 80, Scotsman, Mailand, Italy
Falcon	Falcon™ 15ml, SARSTEDT, Nümbrecht, Germany
Falcon	Falcon™ 50ml, SARSTEDT, Nümbrecht, Germany
Gauze swaps (strainer)	ES-Kompressen, 5x5 cm, 17 threads, 12 layers, Paul Hartmann AG, Germany
ph-meter	Digital-pH-Meter 646, Knick Elektronische Messgeräte GmbH & Co. KG, Berlin, Germany
Pipet	Pipette Pasteur 7 ml, VWR International, Darmstadt, Germany
Pipet	Pipettensatz Eppendorf Research® 5 µl bis 5000 µl, Eppendorf, Hamburg, Germany
Pipette tip	200 µl Bevelled Tip, Starlabgroup, Hamburg, Germany
Pipette tip	1000 µl Graduated Tip, Starlabgroup, Hamburg, Germany
Pipette tip	10 ml Costar Stripette, Corning Incorporated, New York, USA
Pipette tip	5 µl Graduated Tip, Starlabgroup, Hamburg, Germany
Pipette tip	10 µl Graduated Tip, Starlabgroup, Hamburg, Germany
Pipetus®	Pipetus®, Hirschmann Laborgeräte, Eberstadt, Germany

Plate reader	BioTek Synergy 2, Software Gen5™ Version 1.11, BioTek, Winooski, USA
Potter-Elvehjem	Tissue homogenization, unkown provider
Reaction tube	Safe-Lock-Tubes 1,5 ml, Eppendorf, Hamburg, Germany
Reaction tube	Safe-Lock-Tubes 2,0 ml, Eppendorf, Hamburg, Germany
Respirometer software	782 System Version 5.1, Strathkelvin Instruments, North Lanarkshire, Scotland
Respirometer	MT200 und SI782, Strathkelvin Instruments, System Version 4.4 HID, North Lanarkshire, Scotland
Scale	LA230S, Sartorius, Göttingen, Germany
Scale	EMB 2200-0, KERN & Sohn GmbH, Germany
Statistical Software	GraphPad Prism Version 6.0, GraphPad Software, Inc, La Jolla, USA
Stirrer	IKA® EUROSTAR 20 digital 2000 rpm, IKA®-Werke GmbH & Co. KG, Staufen, Germany
Vortex machine	Vortex Genie Touch Mixer 1, Scientific Industries, New York, USA
96-well plate	Mikrotestplatte 96-Well F, SARSTEDT, Nümbrecht, Germany

7.3 List of chemicals

Note: All chemicals have been in p.a. quality.

1,1,3,3-Tetramethoxypropane	Sigma Aldrich Chemie GmbH, Steinheim, Germany
2-Butanol	Merck, Darmstadt, Germany
3-MOPS	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
ADP	Sigma Aldrich Chemie GmbH, Steinheim, Germany
BSA	Sigma Aldrich Chemie GmbH, Steinheim, Germany
C12H18O	Sigma Aldrich Chemie GmbH, Steinheim, Germany
cOmpleteTM	Protease Inhibitor Cocktail, Roche Life Science Mannheim, Germany
Copper (II) sulfate pentahydrate	Merck, Darmstadt, Germany
Cytochrome C	Sigma Aldrich Chemie GmbH, Steinheim, Germany
DMSO	Sigma Aldrich Chemie GmbH, Steinheim, Germany

EGTA	Carl Roth & Co. KG, Karlsruhe, Germany
Folin-Ciocalteu-reagent	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Indomethacin	Sigma Aldrich Chemie GmbH, Steinheim, Germany
L-Glutamate	Fluka Chemie GmbH, Buchs, Schweiz
L-Malate	SERVA, Heidelberg, Germany
Mannitol	Carl Roth & Co. KG, Karlsruhe, Germany
Oligomycin	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Potassium chloride	Fluka Chemie GmbH, Buchs, Schweiz
Potassium dihydrogen phosphate	Merck, Darmstadt, Germany
Potassium hydroxide	Carl Roth & Co. KG, Karlsruhe, Germany
Potassium sodium tartrate tetrahydrate	Merck, Darmstadt, Germany
Rotenone	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Sodium carbonate	Fluka Chemie GmbH, Buchs, Schweiz
Sodium diphosphate	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Sodium hydroxide	Merck, Darmstadt, Germany
Sodium Phosphate	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Sodium succinate	Sigma Aldrich Chemie GmbH, Steinheim, Germany
Sodium chloride 0.9%	Isotonische Kochsalzlösung, Fresenius Kabi, Germany
Sucrose	Carl Roth GmbH & Co. KG, Karlsruhe, Germany
Trypsin (0.05 % Trypsin-EDTA)	Gibco by Life Technologies, Fisher Scientific, USA

Acknowledgement

Firstly, I would like to express my sincere gratitude to Prof. Dr. Olaf Picker and Dr. Anna Herminghaus for their outright support not only with planning, conducting and writing this thesis, but also for their support while I was having a hard time getting started with my first year of residency at the University of Potsdam.

Secondly, I would like to say a big “thank you” to the laboratory team Ms. Claudia Dohle and Ms. Birgitt Berke for their technical assistance under the leading supervision from the head of the laboratory Prof. Dr. Inge Bauer, who provided a friendly and productive working atmosphere.

I thank all my fellow lab-mates for the support, teamwork, critical discussions and lazy afternoons with a cup of coffee. A great friendship with Anna Herminghaus, Jan-Joshua Ripkens and Max Sonnenschein is the best conclusion and gift from the last years together.

At the end, I would like to express my great respect to my parents and my siblings for their continuous support, love and their friendship.

Thank you all!

Albert Johannes Buitenhuis