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RESEARCH PAPER

Protective effect of exogenous nitrite in postoperative ileus

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BACKGROUND AND PURPOSE

As the pathogenesis of postoperative ileus (POI) involves inflammation and oxidative stress, comparable to ischaemia/reperfusion injury which can be ameliorated with nitrite, we investigated whether nitrite can protect against POI and explored the mechanisms involved.

EXPERIMENTAL APPROACH

We used intestinal manipulation (IM) of the small intestine to induce POI in C57BL/6J mice. Sodium nitrite (48 nmol) was administered intravenously just before IM. Intestinal transit was assessed using fluorescent imaging. Bethanechol-stimulated jejunal circular muscle contractions were measured in organ baths. Inflammatory parameters, neutrophil infiltration, inducible NOS (iNOS) activity, reactive oxygen species (ROS) levels, mitochondrial complex I activity and cGMP were measured in the intestinal muscularis.

KEY RESULTS

Pre-treatment with nitrite markedly improved the delay in intestinal transit and restored the reduced intestinal contractility observed 24 h following IM. This was accompanied by reduced protein levels of TNF-α, IL-6 and the chemokine CCL2, along with reduced iNOS activity and ROS levels. The associated neutrophil influx at 24 h was not influenced by nitrite. IM reduced mitochondrial complex I activity and cGMP levels; treatment with nitrite increased cGMP levels. Pre-treatment with the NO scavenger carboxy-PTIO or the soluble guanylyl cyclase inhibitor ODQ abolished nitrite-induced protective effects.

CONCLUSIONS AND IMPLICATIONS

Exogenous nitrite deserves further investigation as a possible treatment for POI. Nitrite-induced protection of POI in mice was dependent on NO and this effect was not related to inhibition of mitochondrial complex I, but did involve activation of soluble guanylyl cyclase.

Abbreviations

GC, geometric centre; I/R, ischaemia/reperfusion; ICAM-1, intercellular adhesion molecule-1; IM, intestinal manipulation; L-012, 8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione sodium salt; MPO, myeloperoxidase; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one; POI, postoperative ileus; PTIO, 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; ROS, reactive oxygen species; sGC, soluble guanylyl cyclase; XOR, xanthine oxidoreductase

Tables of Links

TARGETS
Enzymes
iNOS
MPO, myeloperoxidase
sGC, soluble guanylyl cyclase
XOR, xanthine oxidoreductase

LIGANDS	
Bethanechol	IL-6
CCL2	ODQ
cGMP	TNF-α

These Tables list key protein targets and ligands in this article which are hyperlinked to corresponding entries in http://www.guidetopharmacology.org, the common portal for data from the IUPHAR/BPS Guide to PHARMACOLOGY (Pawson *et al.*, 2014) and are permanently archived in the Concise Guide to PHARMACOLOGY 2013/14 (Alexander *et al.*, 2013).

Introduction

Postoperative ileus (POI) is a transient impairment of gastrointestinal motility, commonly seen after abdominal surgery. It usually resolves within 3 days, but when prolonged, it can lead to increased morbidity, prolonged hospitalization and increased healthcare cost (Kehlet and Holte, 2001). The pathophysiology of POI is marked by an acute neurogenic phase followed by a prolonged inflammatory phase (Boeckxstaens and de Jonge, 2009). The inflammatory phase is characterized by the activation of resident macrophages in the muscular layer, which release inflammatory cytokines such as TNF-α and IL-6, chemokines such as CCL2 and adhesion molecules such as intercellular adhesion molecule-1 (ICAM-1). CCL2 and ICAM-1 will recruit circulatory leukocytes that together with the activated resident macrophages will enhance release of NO through inducible NOS (iNOS). NO has potent inhibitory effects on gastrointestinal motility and causes ileus (Bauer and Boeckxstaens, 2004; Turler *et al.*, 2006). Additionally, reactive oxygen species (ROS) might contribute to POI; our group previously reported an increase in intestinal oxidative stress starting shortly after intestinal manipulation (IM) (De Backer *et al.*, 2009).

Exogenous administration of nitrite was shown to protect heart, liver, kidney and brain from ischaemia/reperfusion (I/R) injury (Duranski *et al.*, 2005; Jung *et al.*, 2006; Shiva *et al.*, 2007; Tripatara *et al.*, 2007). The main mechanisms underlying I/R injury include the generation of ROS and the activation of an inflammatory cascade; both mechanisms make cells more susceptible to cell death (Sanada *et al.*, 2011). The exact mechanism of the protective effect of nitrite in I/R models is not completely understood. Although iNOS-derived NO contributes to inflammatory damage in I/R injury (Iadecola *et al.*, 1995; Wang *et al.*, 2003) and selective iNOS inhibitors can prevent I/R injury (Barocelli *et al.*, 2006) – similar to POI (Kalff *et al.*, 2000; Turler *et al.*, 2006) – evidence suggests that exogenous nitrite needs to be reduced to NO to become effective as the NO-scavengers PTIO (2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide) and carboxy-PTIO prevent the beneficial effect of nitrite (Duranski *et al.*, 2005; Shiva *et al.*, 2007). Beneficial effects might be dependent on providing sufficient NO at areas with a shortage due to deficiency of the two constitutive NOSs (endothelial and neuronal), which under conditions of hypoxia, cannot produce NO. Nitrite is unique in that it will be reduced to NO preferentially under hypoxic conditions and might thus provide NO where needed (Raat *et al.*, 2009). This can less systematically be obtained with NO donors which were shown to induce beneficial (Lozano *et al.*, 2005; Li *et al.*, 2009), no (Hoshida *et al.*, 1996; Zhu *et al.*, 1996) or even detrimental (Mori *et al.*, 1998) effects in I/R models.

Two possible mechanisms of action have been suggested in the protective effect of nitrite-derived NO against I/R injury. Shiva *et al.* (2007) showed in a hepatic I/R model that nitrite can lead to reversible inhibition of mitochondrial complex I by S-nitrosation. Such inhibition of mitochondrial complex I dampens the electron transfer and limits mitochondrial ROS production (Lesnefsky *et al.*, 2004; Shiva *et al.*, 2007). Inhibition of mitochondrial complex I as a pathway for the nitrite-induced protective effect was also described in a cardiac I/R model (Dezfulian *et al.*, 2009). In contrast, Duranski *et al.* (2005) showed in a model of hepatic I/R that nitrite protection was dependent on signalling via soluble guanylyl cyclase (sGC), as it was completely abolished by the sGC inhibitor ODQ (1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one). An sGC-dependent protective effect of nitrite was also suggested in a model of TNF-induced sepsis, in which TNF is known to cause inflammation accompanied by oxidative stress. Treatment with nitrite decreased oxidative stress, mitochondrial damage and mortality, and this protection by nitrite was largely abolished in $sGC\alpha_1$-knockout mice (Cauwels *et al.*, 2009).

Treatment of POI remains mostly supportive, and no real treatment or prevention currently exists. As the pathogenesis of POI also involves inflammation and oxidative stress, components comparable to those in I/R injury which can be counteracted with nitrite, the aim of this study was to investigate whether nitrite can protect against POI and to elucidate the mechanisms involved.

Methods

Animals

All animal care and experimental procedures were approved by the Ethical Committee for Animal Experiments from the

Faculty of Medicine and Health Sciences at Ghent University. The ARRIVE guidelines and the BJP editorial on how these ARRIVE guidelines apply to pharmacological studies were consulted (Kilkenny *et al.*, 2010; McGrath *et al.*, 2010). Male C57BL/6J mice (20–25 g, $n = 249$) were purchased from Janvier, Le Genest St-Isle, France. Mice were housed in an animal care facility with a 12 h light/12 h dark cycle and had free access to water and commercially available chow.

Hepatic I/R model

The hepatic I/R protocol has been described previously (Duranski *et al.*, 2005); more details can be found in the Supporting Information Appendix S1.

POI model

Mice were anesthetized with inhaled isoflurane (5% induction and 2% maintenance) and the abdomen was opened by midline laparotomy. POI was induced by compressing the eventrated small intestine by using sterile moist cotton applicators for 5 min. A fixed dose of sodium nitrite (48 nmol in 50 μL of PBS per mouse; weight range 20-25g) or its solvent (PBS) was given as a bolus injection into the inferior vena cava just before IM. After IM, the bowel was replaced in the abdominal cavity, and the incision was closed by two layers of continuous sutures. Mice were killed by cervical dislocation 6 or 24 h after surgery, and the gastrointestinal tract was removed. Non-operated mice served as controls.

In an additional set of experiments, we studied the influence of the NO scavenger carboxy-PTIO (1 mg kg^{-1} in PBS; injection volume 2.5 μL g^{-1}; i.p. 30 min before IM) and the sGC inhibitor ODQ (20 mg kg^{-1} in DMSO; injection volume 2.5 μL g^{-1}; i.p. 30 min before IM) and its solvent DMSO on nitrite-mediated effects in IM mice. Mice were killed by cervical dislocation 24 h after surgery, and the gastrointestinal tract was removed. In addition, the possible influence *per se* on transit of i.p. administration of carboxy-PTIO and ODQ was investigated in non-operated control mice.

In a final set of experiments, sham-operated animals that underwent laparotomy without IM were compared with non-operated control mice; the possible influence of administration of nitrite into the inferior vena cava in sham-operated mice was also investigated. After measuring transit, the small intestine was flushed with aerated (5% CO_2 in O_2) ice-cold Krebs solution (composition in mM: NaCl 118.5, KCl 4.8, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 1.9, $NaHCO_3$ 25.0 and glucose 10.1) containing 1 mM PMSF and divided into six segments. In the mice killed 24 h after surgery, one segment was used to test the contractile response to bethanechol (see below); in the rest of the segments, the mucosa was removed using a glass slide and the muscularis was stored at −80°C until further processing.

Evaluation of intestinal motility

Intestinal transit [geometric centre (GC)] was evaluated 24 h postoperatively using fluorescence imaging, as described previously (De Backer *et al.*, 2008), and contractile activity was evaluated using the muscarinic agonist bethanechol. A detailed description of both methods can be found in the Supporting Information Appendix S1.

cGMP analysis

cGMP in the mucosa-free segments of the small intestine was extracted and quantified using an enzyme immunoassay kit (Cayman Chemical, Michigan, USA); see Supporting Information Appendix S1 for details.

Mitochondrial isolation and complex I activity

Mitochondria in the mucosa-free segments of the small intestine were isolated as described by Gadicherla *et al.* (2012), and complex I activity was determined by monitoring the change in transmittance from oxidation of NADH to NAD^+ at 340 nm (FLUOstar, BMG Labtech, Ortenberg, Germany); see Supporting Information Appendix S1 for details.

Protein expression levels of CCL2, IL-6 and TNF-α

Protein expression levels of CCL2, IL-6 and TNF-α in the mucosa-free segments of the small intestine were determined by ELISA, according to the manufacturer's protocol (Invitrogen, Merelbeke, Belgium); see Supporting Information Appendix S1 for details.

Neutrophil infiltration

Myeloperoxidase (MPO) activity in the mucosa-free segments of the small intestine was measured as an index of neutrophil infiltration and was based on a previously described protocol (de Jonge *et al.*, 2003); see Supporting Information Appendix S1 for details.

iNOS activity

Inducible NOS enzyme activity in the mucosa-free segments of the small intestine was assayed by measuring the conversion of [^{3}H]-arginine to [^{3}H]-citrulline using an NOS activity assay kit (Cayman Chemical, Michigan, USA), which can be used to measure only iNOS by conducting the assay in calcium-free conditions (Supporting Information Appendix S1).

Reactive oxygen metabolites

Reactive oxygen species levels in the mucosa-free segments of the small intestine were quantified using the luminol derivative L-012 (8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4 (2H,3H)dione sodium salt), as described previously (Castier *et al.*, 2005). L-012 reacts with superoxide, hydrogen peroxide and peroxynitrite (Daiber *et al.*, 2004); see Supporting Information Appendix S1 for details.

Plasma and tissue nitrite concentrations

A group of mice was used to determine the concentration of nitrite in plasma and mucosa-free segments of the small intestine. Mice were anesthetized with inhaled isoflurane (5% induction and 2% maintenance), and blood was taken from the orbital plexus in heparinized tubes to obtain plasma by centrifugation (750 *g*, 2 min, 4°C). A small intestinal sample was taken immediately thereafter. Nitrite or its solvent was administered into the inferior vena cava; plasma and small intestinal samples were taken at 5, 15 and 45 min, and 24 h 15 min after nitrite or solvent administration. These time points correspond to just before IM, immediately after IM,

and 30 min and 24 h after IM. Samples were also obtained from untreated non-operated controls.

Nitrite levels in plasma and in mucosa-free segments of the small intestine were determined by tri-iodide-based gas-phase reductive chemiluminescence (MacArthur *et al.*, 2007). The intestinal tissue samples (0.5 mg tissue per mL buffer) were first homogenized in a buffer containing KCN (1mM), $K_4Fe(CN)_6$ (1 mM), DTPA (diethylene triamine pentaacetic acid; 100 μM) and 1% Nonidet P-40 detergent in PBS (pH 7.4). See Supporting Information Appendix S1 for further details.

Data analysis

All results are expressed as means ± SEM. *n* refers to tissues obtained from different animals. Statistical analysis was performed using a one-way ANOVA followed by Bonferroni's multiple comparison *t*-test or an unpaired Student's *t*-test when only two sets of results had to be compared. A *P*-value less than 0.05 was considered to be statistically significant (Graphpad, San Diego, CA, USA). Intra-batch and inter-batch coefficients of variation (in percent) of the assays performed can be found in the Supporting Information Appendix S2 (Supplementary Table 1).

Materials

The following drugs were used: carbamyl-β-methylcholine chloride (bethanechol), 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide potassium salt (carboxy-PTIO), PMSF, rotenone, sodium nitrite, ubiquinone (CoQ_1) (all obtained from Sigma-Aldrich, Diegem, Belgium), carbachol (Fluka AG, Diegem, Belgium), fluorescein-labelled dextran (70 kDa; Invitrogen, Merelbeke, Belgium), L-012 (Wako Pure Chemical Industries Ltd., Osaka, Japan) and ODQ (Tocris Cookson, Bristol, UK). All drugs were dissolved in de-ionized water except for the following: sodium nitrite and carboxy-PTIO in 10 mM PBS (pH 7.4) and ODQ in DMSO.

Results

Effect of nitrite on IM-induced intestinal dysmotility

In non-operated control mice, fluorescein-labelled dextran (70 kDa) moved to the distal part of the small bowel, whereas after the IM procedure, fluorescein-labelled dextran was retained in the proximal part of the small bowel (Figure 1A); this delay in intestinal transit was quantified by a significant reduction in the GC value of transit (Figure 1B). Nitrite was used at a fixed i.v. dose of 48 nmol per mouse. This dose was reported as most effective in a study on hepatic I/R (Duranski *et al.*, 2005) and in a preliminary series of experiments, we confirmed the pronounced protective effect of 48 nmol nitrite in hepatic I/R (Supporting Information Appendix S2 Supplementary Figure 1). Pre-treatment with this dose of nitrite reduced the IM-induced delay in transit, as indicated by a significant increase in transit GC values (Figure 1A and B). Sham-operated mice did not show a delayed transit compared with non-operated controls, and administration of nitrite to sham-operated mice did not have a significant effect on gastrointestinal motility (Supporting Information Appendix S2 Supplementary Figure 2).

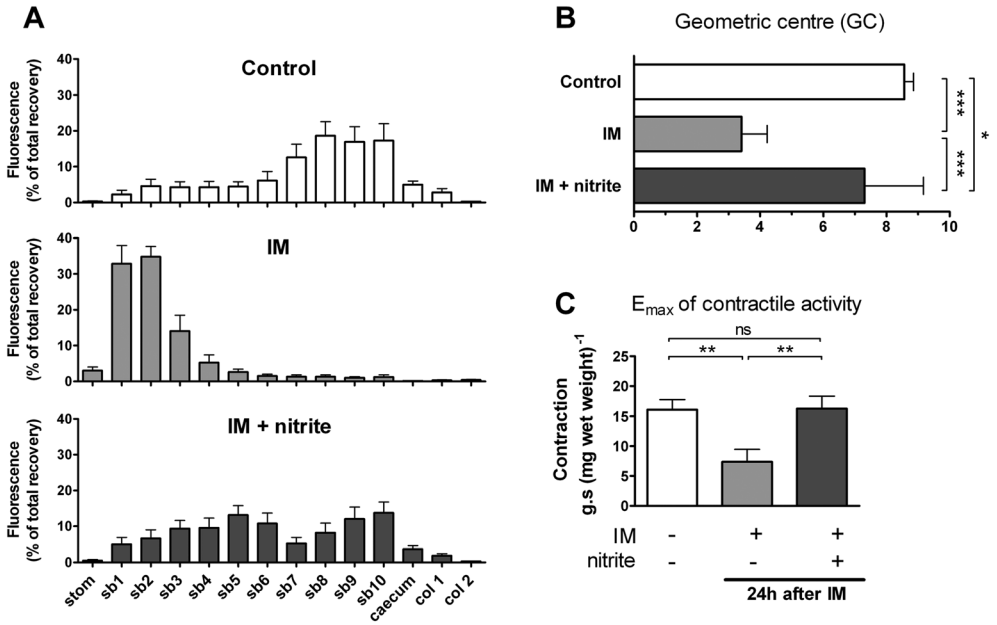

Figure 1

Transit histograms (A) and derived GC values (B) for the distribution of fluorescein-labelled dextran (70 kDa) along the gastrointestinal tract (stom, stomach; sb, small bowel segments; col, colon segments), measured 24 h after IM. E_{max} of bethanechol-stimulated (cumulative 0.3–300 μM; 2 min interval) concentration–response curves of jejunal circular muscle contractile activity (C). Data represent the means ± SEM; $n = 14–15$. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$: one-way ANOVA followed by a Bonferroni multiple comparison test.

The inhibition of intestinal transit after IM reflects inhibited smooth muscle contractile activity of the small intestine; compared with controls, IM caused a reduction in cholinergic contractile activity, indicated by a significantly reduced E_{max} of the cumulative concentration–response curve of bethanechol in jejunal smooth muscle strips. The contractile activity of smooth muscle strips of IM mice, which were pre-treated with nitrite, was restored to that of non-IM control mice (Figure 1C).

Effect of nitrite on IM-induced inflammation and oxidative stress

The inflammatory cytokine TNF-α was not increased at 6 h after IM, but was significantly increased at 24 h after IM. Pre-treatment with nitrite reduced this IM-induced increase at 24 h to TNF-α levels no longer significantly different from those in non-operated control mice (Figure 2A). The inflammatory cytokine IL-6 was significantly increased at both 6 and 24 h after surgery and pre-treatment with nitrite reduced the IM-induced increase in cytokine release at both time points (Figure 2B). The chemokine CCL2 was also significantly increased at both 6 and 24 h after IM. Pre-treatment with nitrite only reduced the IM-induced increase in chemokine release at 24 h. At 6 h after surgery, CCL2 protein levels were 40% higher than when measured 24 h after IM, but this IM-induced increase in CCL2 levels could not be reduced by nitrite (Figure 2C). Neutrophil recruitment (MPO) into the muscularis was significantly increased at 6 and 24 h after IM; compared with 6 h after IM, the influx of neutrophils was doubled at 24 h. Surprisingly, pre-treatment with nitrite markedly reduced the neutrophil infiltration at 6 h, but not at 24 h after IM (Figure 2D).

iNOS activity was not significantly increased at 6 h and was significantly increased at 24 h after IM. Pre-treatment with nitrite reduced this IM-induced elevation in iNOS activity to levels of iNOS activity no longer significantly different from those in non-operated control mice (Figure 2E). Levels of ROS, as measured with the chemiluminescent dye L-012, were not increased at 6 h after IM, but were markedly increased 24 h after surgery. Pre-treatment with nitrite reduced this IM-induced increase to ROS levels not different from those in non-operated controls (Figure 2F).

Investigation of the possible role of mitochondrial complex I and sGC in the effect of nitrite

Mitochondrial complex I activity was significantly reduced 6 and 24 h after IM. Pre-treatment with nitrite did not influence this reduction in enzyme activity after IM (Figure 3A). cGMP levels in the intestinal muscularis were significantly reduced 6 and 24 h after IM. Pre-treatment with nitrite increased these reduced cGMP levels after IM significantly at 6 h, and to cGMP levels no lower than those of non-operated controls at 24 h (Figure 3B).

These results suggest that the nitrite-induced protective effect in POI might be dependent on sGC activation. This was further elaborated by exploring the influence of the sGC inhibitor ODQ on nitrite-mediated protective effects. Administration of ODQ (which did not have an influence *per se* on transit in control mice; Supporting Information

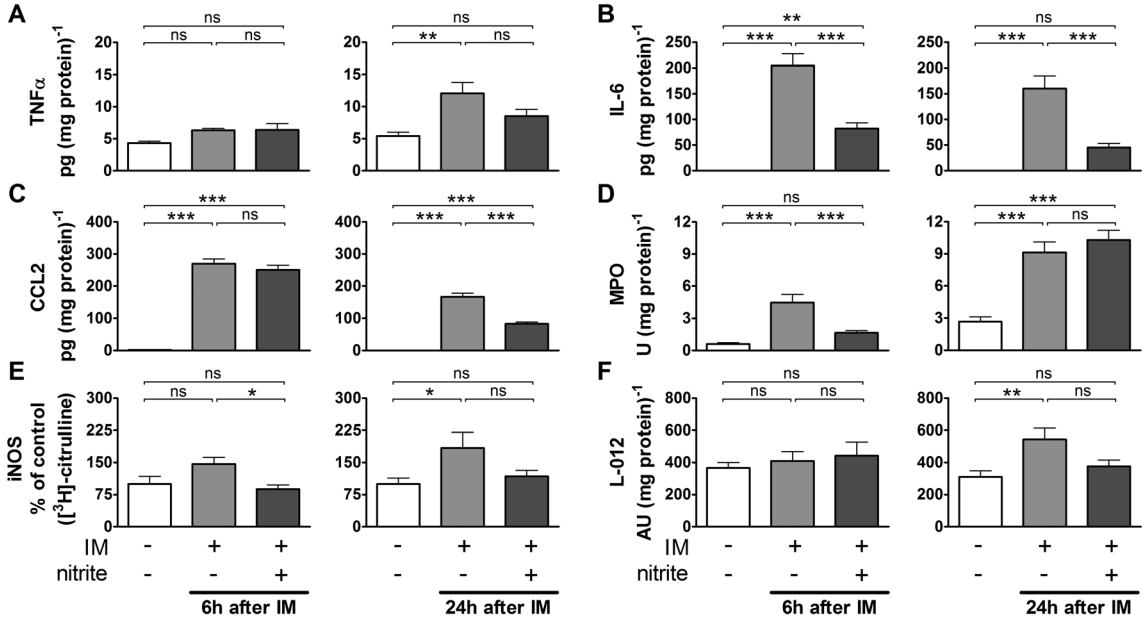

Figure 2

Effect of nitrite on IM-induced changes in TNF-α (A), IL-6 (B) and CCL2 (C) protein levels, in neutrophil infiltration (D; MPO), in iNOS enzyme activity (E) and in ROS levels (F; assessed with the luminol derivate L-012), measured 6 and 24 h after IM. Values at 6 h were obtained in a separate series of experiments with its own non-manipulated control group. Data represent the means ± SEM; $n = 6$–10. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$: one-way ANOVA followed by a Bonferroni multiple comparison test. The blank columns represent readings below the detection limit of the assay (i.e. 2.0 pg (mg protein)$^{-1}$ for IL-6 and 4.7 pg (mg protein)$^{-1}$ for CCL2).

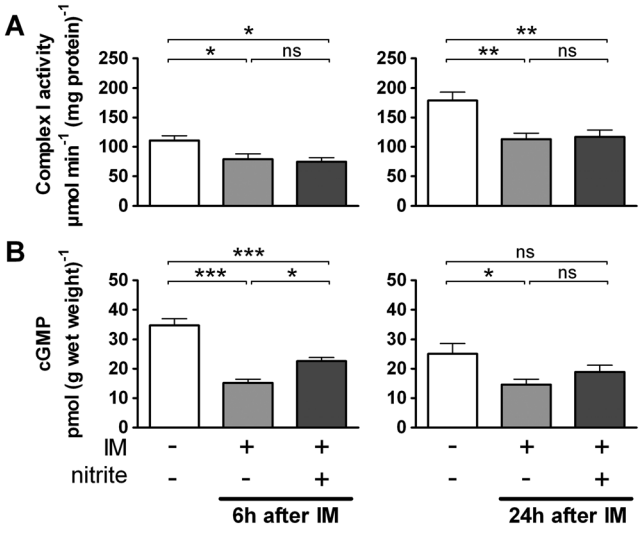

Figure 3

Effect of nitrite on IM-induced changes in mitochondrial complex I activity (A) and cGMP levels (B), measured 6 and 24 h after IM. Values at 6 h were obtained in a separate series of experiments with its own non-manipulated control group. Data represent the means ± SEM; $n = 8$. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$: one-way ANOVA followed by a Bonferroni multiple comparison test.

Appendix S2 Supplementary Figure 3) completely prevented the accelerating effect of nitrite on delayed transit in IM mice, as shown by the reduction in GC to a level comparable with that in non-treated IM mice (Figure 4A). Correspondingly, the cholinergic contractile activity of jejunal smooth muscle strips was restored to the level of non-treated IM mice (Figure 4A and B).

Inflammatory parameters (Figure 5A–C) and ROS levels (Figure 5D) were increased, significantly for CCL2 and iNOS, and non-significantly for TNF-α and L-012, after pre-treating nitrite-treated IM mice with ODQ. In addition, pre-treatment with ODQ prevented the nitrite-induced increase in cGMP levels in manipulated mice (Figure 5E). The vehicle for ODQ, DMSO, which was tested in parallel in nitrite-treated IM mice, to exclude the possibility that DMSO *per se* could affect the protective effects of nitrite, did not influence the results of nitrite treatemtn (data not shown; $n = 6$).

Influence of the NO scavenger carboxy-PTIO on nitrite-induced protection

The protective effects of nitrite in POI appear to be NO dependent, as the NO scavenger carboxy-PTIO (which did not have an influence *per se* on transit in control mice; Supporting Information Appendix S2 Supplementary Figure 3) completely inhibited the nitrite-induced protection on gastrointestinal motility in IM mice (Figure 4). As observed with ODQ, pre-treatment with carboxy-PTIO increased inflammatory parameters (Figure 5A–C) and ROS levels (Figure 5D), significantly for CCL2 and iNOS, and non-significantly for TNF-α and L-012, when comparing them with those of nitrite-treated IM mice. The nitrite-induced increase in cGMP levels of IM mice was also prevented by carboxy-PTIO.

Nitrite levels in plasma and small intestine

The nitrite level in plasma was 0.89 ± 0.17 µM ($n = 6$) in non-operated control mice (not shown in Figure 6). In mice receiving the solvent of nitrite, the plasma concentration of nitrite remained at the control level at all time points of measurement (Figure 6A). Immediately after administration of 48 nmol of nitrite, the plasma level increased to 18.3 ± 3.1 µM ($n = 6$), quickly declining thereafter but being still significantly higher than in mice treated with solvent immediately after and 30 min after IM. At 24 h after IM, the nitrite level in plasma had returned to the basal control level.

The nitrite level in mucosa-free segments of the small intestine of non-operated control mice was 0.028 ± 0.004 µmol mg^{-1} protein. In operated mice treated with the solvent of nitrite, the small intestinal nitrite concentration remained at a similar level at all time points of measurement

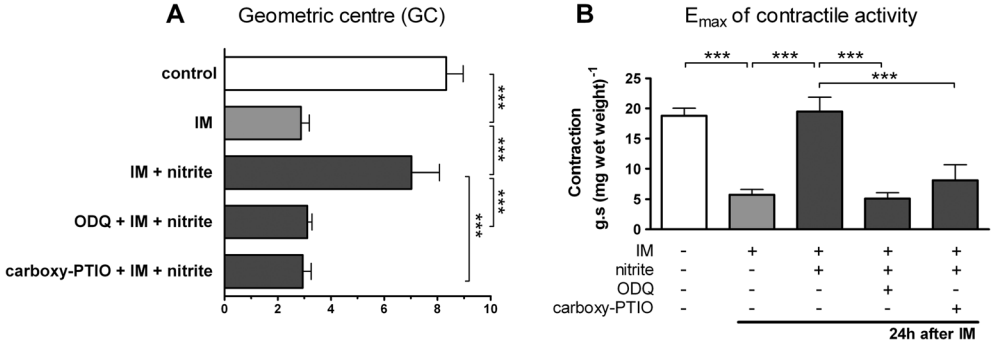

Figure 4

Effects of the sGC inhibitor ODQ and the NO scavenger carboxy-PTIO on nitrite-induced protection against manipulation-induced intestinal dysmotility. Transit GC values (A) for the distribution of fluorescein-labelled dextran (70 kDa) along the gastrointestinal tract, measured 24 h after IM and E_{max} of bethanechol-stimulated (cumulative 0.3–300 µM; 2 min interval) concentration–response curves of jejunal circular muscle contractile activity (B). Data represent the means ± SEM; $n = 6$–8. ***$P < 0.001$: one-way ANOVA followed by a Bonferroni multiple comparison test.

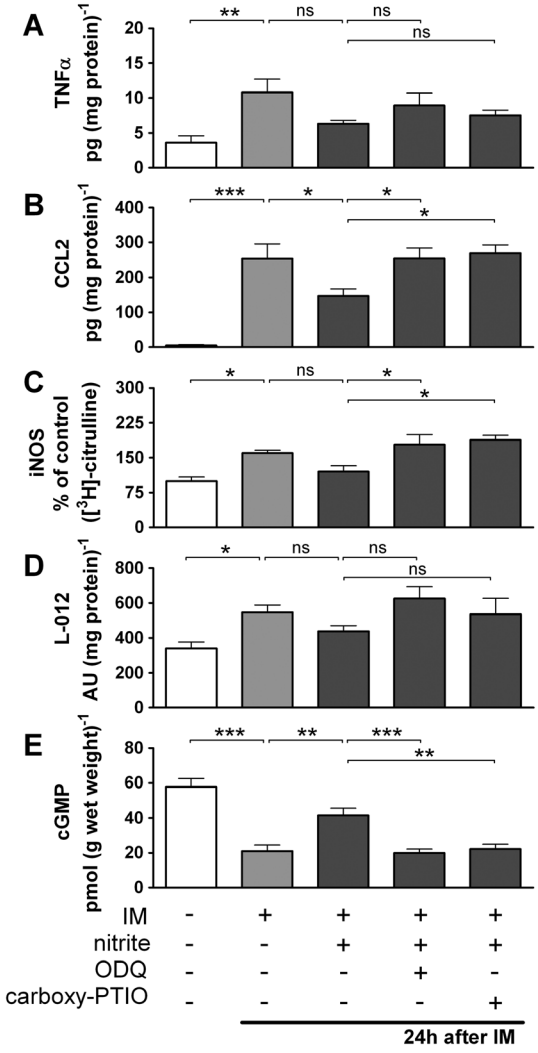

Figure 5

Effects of the sGC inhibitor ODQ and the NO scavenger carboxy-PTIO on the effect of nitrite versus IM-induced changes in TNF-α (A) and CCL2 protein levels (B), in iNOS enzyme activity (C), in ROS levels (D; assessed with the luminol derivative L-012) and in cGMP levels (E), measured 24 h after IM. Data represent the means ± SEM; $n = 5–8$. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$: one-way ANOVA followed by a Bonferroni multiple comparison test.

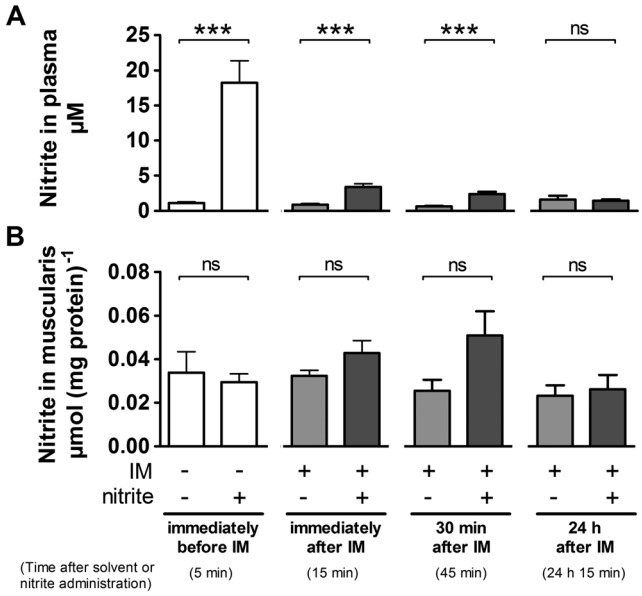

Figure 6

Nitrite levels in plasma (A) and mucosa-free segments of the small intestine (B) after nitrite (48 nmol) or solvent administration. Nitrite measurements were carried out at different time points: immediately before IM (i.e. 5 min after intravenous administration of solvent or nitrite), immediately after IM (i.e. 15 min after intravenous administration of solvent or nitrite) and 30 min (i.e. 45 min after intravenous administration of solvent or nitrite) or 24 h (i.e. 24 h 15 min after intravenous administration of solvent or nitrite) after IM. Data represent the means ± SEM; $n = 6$. $^{***}P < 0.001$: unpaired Student's *t*-test.

(Figure 6B). In mice receiving nitrite intravenously, there was a trend towards increased levels of nitrite in the small intestine immediately after and at 30 min after IM (Figure 6B).

Discussion and conclusions

Ileus, a transient impairment of gastrointestinal motility, is a common complication seen after abdominal surgery for which there is no single preventive treatment. As the pathogenesis of POI involves inflammation and oxidative stress, and thus resembles I/R injury which can be improved with nitrite, we investigated whether nitrite could also protect against POI.

The inflammatory response triggered by handling of the intestine is now generally accepted as a key event in POI (Bauer and Boeckxstaens, 2004; Boeckxstaens and de Jonge, 2009). Surgical manipulation of the small intestine activates the resident macrophages in the muscularis externa, resulting in the release of macrophage-derived cytokines, chemokines and adhesion molecules (Wehner *et al.*, 2007). This local release of pro-inflammatory molecules is followed by a cellular inflammatory response with extravasation of circulatory leukocytes – mainly neutrophils and monocytes – into the intestinal muscularis (Kalff *et al.*, 1998; Kalff *et al.*, 1999). iNOS expressed in recruited and resident leukocytes will then generate NO, which directly modulates the contractile activity of the muscularis, contributing to inhibition of gastrointestinal transit and POI (Kalff *et al.*, 2000; Turler *et al.*, 2006). Similarly, when manipulating the murine intestine in the present study, this was followed by (1) an increase in inflammatory cytokines and chemokines, (2) an influx of neutrophils and (3) an increase in iNOS activity in the intestinal muscularis. For the increased levels of the chemokine CCL2 and of MPO, a marker of neutrophil influx, a particular time course was observed. Compared to 6 h after IM, CCL2 levels were reduced by 40%, and neutrophil influx was doubled at 24 h after IM, corresponding to the time course for CCL2 and MPO levels reported in previous studies on POI in rodents (de Jonge *et al.*, 2003; Wehner *et al.*, 2007; Schmidt *et al.*, 2012).

The extent of intestinal dysmotility is known to be proportional to the level of intestinal inflammation (Kalff *et al.*, 1998), and prevention or reduction of the manipulation-induced inflammatory response by, for example, inhibition of macrophage function or inhibition of leukocyte infiltration by ICAM-1 blockade attenuated dysmotility (The *et al.*, 2005; Wehner *et al.*, 2007). In accordance, we showed that administration of nitrite effectively accelerated the IM-induced delay in gastrointestinal transit corresponding with suppression of the inflammatory response, as shown by a reduction in the inflammatory cytokines TNF-α and IL-6 and in CCL2 levels by 24 h after IM. The pronounced increase in IL-6 at 6 h was also suppressed by nitrite. Surprisingly, at 6 h after IM, increased levels of the chemokine CCL2, known to play an essential role in the recruitment of monocytes to sites of injury in several inflammatory models (Lu *et al.*, 1998), were not reduced by nitrite. This is in contrast with the results for MPO, a marker for neutrophil influx, which was reduced by nitrite at 6 h but not at 24 h after IM although the IM-induced decrease of gastrointestinal transit and the associated reduced contractile activity were almost completely restored by nitrite 24 h after IM. We do not have an explanation for this time-related difference in the effects of nitrite on monocytes and neutrophils, but some degree of reduced monocyte infiltration and of delayed neutrophil infiltration seems involved in the protective effect of nitrite.

The ROS generated in inflammation might also contribute to POI. Anup *et al*. (1999) reported that surgical manipulation of the rat intestine resulted in an increase of activity of one of the few ROS-generating enzyme systems, xanthine oxidase, in the enterocytes. This was associated with widened intercellular spaces and increased mucosal permeability; changes that were prevented by pre-treatment of the animals with xanthine oxidase inhibitors (Anup *et al.*, 1999; Anup *et al.*, 2000). In addition, our group previously reported an increase in oxidative stress in mouse small intestine after IM; reducing oxidative stress (with the CO-releasing molecule CORM-3) correlated with a positive effect on postoperative intestinal transit (De Backer *et al.*, 2009). In the present study, we measured an increase in ROS in the intestinal muscularis 24 h after IM, which was attenuated by nitrite and might contribute to its beneficial effect on ileus. In line with our results, antioxidant effects of nitrite were also demonstrated in an I/R model of the brain and in an ischaemic model of the heart, associated with protection against I/R injury (Jung *et al.*, 2006; Singh *et al.*, 2012).

Nitrite will be reduced to NO under hypoxic conditions (Raat *et al.*, 2009). This concept led to studies testing nitrite as an NO donor in experimental I/R models of the heart, liver, kidney and brain (Duranski *et al.*, 2005; Jung *et al.*, 2006; Shiva *et al.*, 2007; Tripatara *et al.*, 2007). Nitrite will provide NO at the time and location needed, demonstrating its advantage over the classical NO donors that have yielded conflicting results in previous I/R studies, probably due to their lack of 'specificity' (Hoshida *et al.*, 1996; Zhu *et al.*, 1996; Mori *et al.*, 1998; Lozano *et al.*, 2005; Li *et al.*, 2009). The critical role for nitrite-derived NO in I/R models was apparent from the fact that the protective effects of nitrite were abolished in the presence of an NO scavenger (Duranski *et al.*, 2005; Jung *et al.*, 2006; Shiva *et al.*, 2007; Tripatara *et al.*, 2007). In the present study, administration of the NO scavenger carboxy-PTIO completely inhibited the nitrite-induced protection of gastrointestinal dysmotility after IM and prevented the nitrite-induced reduction of IM-induced inflammation and oxidative stress. This supports the idea of a mechanism requiring the reduction of nitrite to NO to protect against POI. This reduction might be related to temporarily decreased oxygen levels in the intestine, due to repetitive momentary ischaemia by IM. The fact that the increase in nitrite levels seen in plasma just after the administration of nitrite was greatly reduced shortly after IM would support this idea.

The basal level of nitrite (0.89 μM) was similar to basal nitrite levels reported for mouse plasma [0.97 μM (Duranski *et al.*, 2005), 0.79 μM (Dezfulian *et al.*, 2009) and 0.70 μM (Shiva *et al.*, 2006)]. As early as 5 min after i.v. administration of nitrite, the plasma level attained 18.3 μM. In humans, nitrite has a half-life of 10–13 min (Giustarini *et al.*, 2004; Dejam *et al.*, 2005; Tsikas, 2005) and, extrapolating this half-life to mice, the nitrite level should decrease from 18.3 μM to approximately 9 μM from 5 to 15 min after nitrite administration. However, a plasma nitrite level of 3.4 μM was measured at 15 min after nitrite administration in operated animals, indicating that the decrease in nitrite level over this 10 min period is more pronounced than expected from the half-life. Part of the nitrite might be taken up by the manipulated intestine. Indeed, the decrease in nitrite levels in plasma immediately after and even more at 30 min after IM was associated with an increase in nitrite levels in the intestinal muscularis.This might indicate that the muscularis indeed uses circulating nitrite from the plasma as a storage pool for NO to exert its protective effect. It seems remarkable that a single i.v. dose of nitrite can influence the depressed gut motility at 24 h after surgery. However, the delay in transit observed 24 h after IM is due to mechanisms initiated during IM and progressing thereafter. If the start and the progression of these mechanisms is suppressed by one injection of a substance – even with a short half-life – just before the IM, then this will also affect the results at 24 h.

Which nitrite reductases reduce nitrite to NO in ischaemic conditions is currently an area of intense research. In myocardial I/R injury, protection against myocardial infarction by nitrite was absent in myoglobin knockout mice, which supports the hypothesis that myoglobin serves a critical function as an intrinsic nitrite reductase, regulating cellular responses to hypoxia (Hendgen-Cotta *et al.*, 2008). In hepatic and renal I/R injury, however, xanthine oxidoreductase (XOR) was shown to play an essential role in the enzymic conversion of nitrite to NO as the XOR inhibitor allopurinol attenuated the protective effect of nitrite-derived NO in these models (Lu *et al.*, 2005; Tripatara *et al.*, 2007). In addition, in an rat isolated heart model where nitrite-derived NO protected against the damaging effects of I/R injury, XOR was also shown to be involved in the formation of NO from nitrite (Webb *et al.*, 2004). Furthermore, deoxyhaemoglobin has been implicated in controlling nitrite-dependent NO signalling in the human vasculature during exercise induced stress, tightly regulated by haemoglobin oxygen desaturation (Cosby *et al.*, 2003). It remains to be determined which of these proteins (myoglobin, XOR or deoxyhaemoglobin) is responsible for the reduction of nitrite to NO in the POI model.

To elucidate the mechanism of action of nitrite-derived NO in the protective effect in POI, we explored two possible

mechanisms of action suggested in the protective effect of nitrite-derived NO against I/R injury, namely reversible inhibition of mitochondrial complex I by S-nitrosation (Shiva *et al.*, 2007; Dezfulian *et al.*, 2009) and activation of sGC by NO (Duranski *et al.*, 2005; Jung *et al.*, 2006). In correspondence with I/R studies, mitochondrial complex I activity was significantly decreased after IM, probably due to temporally decreased oxygen levels during the IM procedure, necessary for oxidative phosphorylation. Although nitrite-induced protection by inhibition of the electron transport might seem counterintuitive, the continuation of mitochondrial oxidative phosphorylation in the context of low O_2 generates ROS, mitochondrial calcium overload and the release of cytochrome c (Chen *et al.*, 2007; Shiva *et al.*, 2007). Consequences to the cell include oxidative damage, opening of the mitochondrial permeability transition pore and activation of apoptotic cascades, all favouring cell death. Pre-treatment with nitrite did not influence complex I activity in mice after IM, indicating that nitrite protection in our POI model is not mediated via reversible inhibition of mitochondrial complex I. We therefore focused on a possible mechanism via the NO–sGC–cGMP pathway, as suggested earlier in I/R models of liver and brain, in an ischaemic heart model, and in a model of TNF-induced sepsis (Duranski *et al.*, 2005; Jung *et al.*, 2006; Cauwels *et al.*, 2009; Singh *et al.*, 2012). In correspondence with the findings in the ischaemic heart model where cGMP levels were also measured (Singh *et al.*, 2012), IM significantly decreased cGMP levels in the intestinal muscularis, but pre-treatment with nitrite increased these cGMP levels again, supporting the idea that the protective effect of nitrite in POI might be dependent on sGC, generating cGMP upon activation. The fact that both the NO scavenger carboxy-PTIO and the sGC inhibitor ODQ brought intestinal cGMP levels in nitrite-treated IM mice back to those of non-treated IM mice, and that they prevented nitrite-induced protection on IM-induced intestinal dysmotility and nitrite-induced reduction of IM-induced inflammation and oxidative stress, supports the possibility that the nitrite-induced protection in the POI model was mediated via sGC. The exact mechanism by which the nitrite–NO–sGC–cGMP pathway exerts its protective effects in POI is still to be elucidated. In an I/R model of the brain, the protective effect of nitrite-derived NO via sGC activation was dependent upon its vasodilatory effects (Jung *et al.*, 2006), while in a model of I/R injury in isolated mouse heart (Bell *et al.*, 2003), activation of sGC by an NO donor led to opening of the mitochondrial K_{ATP} channels, thereby preserving mitochondrial function by preventing mitochondrial permeability transition pore opening and cytochrome c release, normally leading to cell death (Korge *et al.*, 2002). The latter might play a role in the effect of nitrite in POI, as enterocyte mitochondrial dysfunction was shown to be associated with surgical manipulation of the intestine and this dysfunction was prevented in the presence of the NOS substrate L-arginine (Anup *et al.*, 2001; Thomas *et al.*, 2001). Nitrite was – via an sGC-dependent pathway and most likely via the generation of NO – also shown to increase mucus thickness in the rat stomach (Bjorne *et al.*, 2004). Because increased mucosal permeability with translocation of intraluminal bacteria and activation of resident macrophages has been implicated in the potentiation of ileus (Snoek *et al.*, 2011), increased production of mucus after nitrite administration might also contribute to the protective effect of nitrite in POI by providing a barrier to luminal content (Allen *et al.*, 1993).

In conclusion, our present results indicate that an intervention with exogenous nitrite deserves further investigation as possible treatment to prevent POI. We have demonstrated that nitrite attenuated POI in mice, corresponding with a reduction in IM-induced inflammation and oxidative stress in the intestinal smooth muscle. Mechanistically, nitrite-induced protection was dependent on reduction to NO and was not associated with inhibition of mitochondrial complex I, but did involve activation of sGC.

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Author contributions

R. L. designed the study. S. C. performed the experiments. S. S. performed the nitrite measurements. S. C. and R. L. analysed the data and wrote the manuscript.

Conflict of interest

Authors declare that they have no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

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Appendix S1 Supplementary Methods.
Appendix S2 Supplementary Results.
Supplementary Table 1 Intra-batch coefficient of variation was determined for the samples obtained 24 h after IM (n = 18–28), measured in duplicate. Inter-batch coefficient of variation was determined by use of the plate control means for control samples with low and high content. For assays not having control samples (*), the inter-batch coefficient of variation was determined from the plate means for the samples of non-operated controls.
Supplementary Figure 1 Confirmation of the protective effect of 48 nmol of nitrite in hepatic I/R injury. Effect of nitrite on serum ALT (A) and AST (B) levels measured after 45 min of hepatic ischaemia and 5 h of reperfusion. Data represent the means ± SEM; n = 6–7. ***$P < 0.001$: one-way ANOVA followed by a Bonferroni multiple comparison test.
Supplementary Figure 2 Comparison of the gastrointestinal motility in non-operated control mice, and in sham-operated mice without or with nitrite treatment. Transit histograms (A) and geometric centre (B) for the distribution of fluorescein-labelled dextran (70 kDa) along the gastrointestinal tract (stom, stomach; sb, small bowel segments; col, colon segments) measured 24 h after IM. Data represent the means ± SEM; n = 6. A one-way ANOVA followed by a Bonferroni multiple comparison test was applied, but no significance was found.
Supplementary Figure 3 Influence of the sGC inhibitor ODQ and the NO scavenger carboxy-PTIO in non-operated control mice. Transit histograms (A) and geometric centre (B) for the distribution of fluorescein-labelled dextran (70 kDa) along the gastrointestinal tract (stom, stomach; sb, small bowel segments; col, colon segments) measured 24 h after IM. Data represent the means ± SEM; n = 6. A one-way ANOVA followed by a Bonferroni multiple comparison test was applied, but no significance was found.