Knock out of neuronal nitric oxide synthase exacerbates intestinal ischemia/reperfusion injury in mice

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Abstract

Recent investigation of the intestine following ischemia and reperfusion (I/R) has revealed that nitric oxide synthase (NOS) neurons are more strongly affected than other neuron types. This implies that NO originating from NOS neurons may contribute to neuronal damage. However, there is also evidence of neuroprotective effects of NO. In this study we compared the effects of I/R on the intestines of neuronal NOS knockout (nNOS^{-/-}) mice and wild-type mice. The damage to the mucosa and muscle was more severe in nNOS^{-/-} mice, and there was a greater infiltration of the external muscle by neutrophils in the first 24 hr. The contractile protein, α -smooth muscle actin, was in the majority of smooth muscle cells in the external musculature of normal mice, but was absent from most cells, and was reduced in the cytoplasm of other cells following I/R. The loss was greater in nNOS^{-/-} mice. Basal contractile activity of the longitudinal muscle and contractile responses to nerve stimulation or a muscarinic agonist were reduced in regions subjected to I/R and the effects were greater in nNOS^{-/-} mice. There were also reductions in responsiveness in regions of operated mice not subjected to I/R. This is attributed to post operative ileus that is not significantly affected by knockout of nNOS. The results indicate that deleterious effects are greater in regions subjected to I/R in mice lacking nNOS compared to normal mice, implying that NO produced by nNOS has protective effects that outweigh any damaging effect of this free radical produced by enteric neurons.

Key words: Ischemia, nitric oxide synthase, enteric nervous system, intestine

Introduction

Nitric oxide (NO) is a free radical involved in numerous biological functions, including vasodilation, transmission from inhibitory neurons to gut muscle, modulation of neurotransmission, inhibition of platelet aggregation, and inhibition of smooth muscle proliferation (Love 1999; Moro et al. 2004). NO is synthesised from L-arginine by the activity of nitric oxide synthases (NOS) (Love 1999; Mallick et al. 2004; Moro et al. 2004). All three isoforms of NOS have been identified in the intestine: endothelial NOS (eNOS), neuronal NOS (nNOS) and inducible NOS (iNOS) (Torihashi et al. 1996; McCafferty et al. 1999; Mallick et al. 2004). Interestingly, neurons containing nitric oxide synthase are more susceptible to damage than other enteric neurons in a number of enteric neuropathies, including esophageal achalasia, hypertrophic pyloric stenosis and gastroparesis (Rivera et al. 2011a).

Damage following ischemia and reperfusion (I/R) injury is common in the intestine, and can be caused during abdominal surgery, in several disease states and following intestinal transplantation (Granger and Korthuis 1995). I/R results in initial tissue damage, and in prolonged changes in motility, most often slowed transit, implying that properties of motility-controlling neurons are altered. We have previously reported that a brief period of ischemia followed by reperfusion causes damage selectively to nNOS neurons (Rivera et al. 2009; Rivera et al. 2011b). nNOS immunoreactive neurons, that have been identified as inhibitory motor neurons and descending interneurons in the mammalian enteric nervous system, were significantly swollen and distorted after 24 hours of reperfusion following a period of ischemia, with the neuronal changes persisting for at least four weeks (Rivera et al. 2009), although changes in the muscle and mucosa were repaired by a week (Pontell et al. 2011). There is also a substantial increase in the occurrence of nitrosylated proteins after I/R (Rivera et al. 2011b). Tyrosine residues in proteins are nitrosylated at position 3 by peroxynitrites that are formed from NO and reactive oxygen species (Ferrer-Sueta and Radi 2009). The nitrosylation compromise protein activity and lead to deficits in cell function. We found extensive accumulations of nitrosylated proteins in the intestine within 1 hr after I/R. The nitrosylated protein was cleared over a period of about 2 days.

The previous investigation of I/R indicated that NO, probably originating from NOS neurons, contributed to protein degradation and cell damage (Rivera et al. 2011b). However, there is good evidence that NO has dual protective and damaging roles when there is

ischemic challenge to the brain (Lipton 1999; Moro et al. 2004; Woodruff et al. 2011) and to the intestine (Miller et al. 1994; Masuda et al. 1995; Pfeiffer and Qiu 1995; McCafferty et al. 1999). The present work was undertaken to investigate the role of neuronally-produced NO in the tissue damage that occurs after I/R, by investigating changes in nNOS knockout (nNOS^{-/-}) mice. We have also investigated changes in neuromuscular function, which has not been previously studied in this model of intestinal I/R.

Materials and Methods

Mice homozygous for targeted disruption of the nNOS gene (Huang et al. 1993) from an established colony at the Department of Anatomy and Neuroscience at the University of Melbourne were used for the experiments. The colony was established by backcrossing nNOS^{-/-} mice, purchased from Jackson Laboratories (Bar Harbor, ME, USA, stock no. 002633) onto a C57BL/6 background. Male and female nNOS^{-/-} and nNOS^{+/+} littermates (8-12 weeks of age) were used in all experiments. All procedures were conducted according to the National Health and Medical Research Council of Australia guidelines and were approved by the University of Melbourne Animal Experimentation Ethics Committee.

Induction of ischemia

Mice were anaesthetised with a mixture of xylazine (20 mg/kg; Troy Laboratories, Sydney, Australia) and ketamine hydrochloride (100 mg/kg; Troy Laboratories) diluted in sterile phosphate buffered saline, given subcutaneously. A loop of ileum (approximately 5 cm long) that was supplied by a single branch of the superior mesenteric artery was exteriorised, ligatures were tightly applied to the marginal arteries and veins to prevent blood flow between the segment subjected to I/R and the adjacent regions, and the mesenteric artery and vein supplying the segment were occluded for 120 min with an atraumatic vascular clamp. Prompt restoration of flow was achieved by dripping lidocaine (1% in saline; Troy Laboratories) on the region of occlusion immediately after removing the clamp.

Animals were taken at 3 and 24 hr after occlusion (at least $n = 3$ in each group). Tissue was taken from the region subjected to ischemia (occluded region) and from a non-ischemic (non-occluded) region, 4-6 cm oral to the ischemic region. Tissue was also taken from sham operated mice and from unoperated mice. Sham operations followed the same procedures, except that the ties on marginal vessels were not tightened, and the atraumatic vascular clamp was not closed. The sham occluded and sham non-occluded regions were taken after 24 hr, this being the time point of maximum effect of I/R.

Histology

At the time of taking tissue, animals were killed by cervical dislocation. The occluded and non-occluded regions were removed and placed in phosphate buffered saline (PBS: 0.15 M NaCl in 0.01 M sodium phosphate buffer, pH 7.2), containing nicardipine (10^{-6} M, Sigma, Sydney, Australia). The ileum was cleaned of contents, opened along the mesenteric attachment and pinned, mucosa down, to balsa wood sheets without stretching for fixation in 2% formaldehyde plus 0.2% picric acid in 0.1M sodium phosphate buffer, pH 7.2, at 4°C. Each sample was placed in a histology cassette and dehydrated through graded ethanol to histolene and embedded in paraffin. Sections were cut transversely at 5 μ m thickness and processed for standard haematoxylin and eosin (H&E) staining. Following staining, tissue was mounted using permanent mounting medium. Preparations were examined on a Zeiss microscope fitted with an Axio-Cam imaging system.

Immunohistochemistry

Occluded and non-occluded segments of intestine were opened along the mesenteric border and pinned mucosa down to balsa wood sheets for fixation in 2% formaldehyde plus 0.2% picric acid in 0.1M sodium phosphate buffer, pH 7.2, at 4°C. Preparations were cleared of fixative by 3 x 10 min washes in dimethyl sulfoxide (DMSO) followed by 3 x 10 min washes in PBS. Fixed tissue was stored at 4°C in PBS containing sodium azide (0.1%) and 30% sucrose as a cryoprotectant (PBS-sucrose-azide). The following day, tissue was transferred to a mixture of PBS-sucrose-azide and OCT compound (Tissue Tek, Elkhart, Ind., USA) at a ratio of 1:1 for a further 24 hr before being embedded in 100% OCT. Sections (12 μ m thick) were cut, collected on 1% gelatinized slides, left to dry for 1hr at room temperature, and then incubated with rabbit anti α -smooth muscle actin (1:1000; Abcam, Cambridge, UK), α -vasointestinal peptide (VIP) (1:400; Furness and Costa 1979) overnight at 4°C. The sections were washed for 3 x 10 min in PBS prior to the secondary antibody incubation with donkey anti-rabbit 488 (Molecular Probes, Eugene, Ore., USA), 1:500 for 1 hr at room temperature. Sections were washed 3 x 10 min in PBS prior to 10 min incubation with DAPI (Invitrogen, Melbourne, Australia), 1:1000, at room temperature. Three subsequent 10 min washes in PBS were given and sections were then mounted using S3023 non-fluorescent mounting medium (Dako Corporation, Carpinteria, CA, USA).

α -Smooth muscle actin immunoreactive cells were counted in the longitudinal muscle layer. Data are expressed as the number of α -smooth muscle actin cells per section, per length of ileum.

Myeloperoxidase reaction

Segments of intestine were opened along the mesenteric border and pinned mucosa down, to balsa wood sheets. The tissue was fixed in 100% ethanol for 10 min. Tissue preparations were then subjected to 3 x 10 min PBS washes. Preparations were stored in PBS-sucrose (PBS containing 30% sucrose) overnight at 4°C. The following day, tissue was transferred in a solution of 50% OCT and 50% PBS-sucrose-azide. On the following day, the tissue was embedded in OCT and snap frozen, using isopentane that was cooled in liquid nitrogen, and stored at -20°C until required. Ileum sections of 12 µm thickness, 12 µm apart, were cut and collected on 1% gelatinized slides and left to dry for 1 hr at room temperature then covered in PBS until ready for staining.

Preparations were then stained in a Hanks-Yates solution consisting of 0.6 mg/mL Hanks-Yates reagent (p-phenylenediamine plus pyrocatechol; Polysciences, Inc., Warrington, PA, USA) and 0.003% H₂O₂ in PBS for 10 min to detect the presence of myeloperoxidase (MPO) in neutrophils (Kalff et al. 1998b). Following 3 x 5 min washes in PBS, tissue was then counterstained with 2% methylene green for 30 seconds. Preparations underwent further washes in ethanol and histolene and were then mounted using DPX.

Neutrophils that were localised using the MPO reaction were counted in three compartments, the longitudinal muscle/ myenteric plexus, the circular muscle and the submucosa/ intestinal glands. Neutrophils observed in the villi were not counted because of the disruption and loss of villi that occurred at early time points. Data are expressed as the number of MPO positive cells per section, per length of ileum.

Isometric tension recording

Full thickness ileal segments (2 cm long) were prepared and mounted longitudinally in 20 mL organ baths containing Krebs' physiological saline solution of the following composition (in mmol/L): 118 NaCl, 4.8 KCl, 2.5 NaHCO₃, 25 NaH₂PO₄, 1.1 MgSO₄, 11.1 D-glucose, 2.5 CaCl₂. The physiological saline also contained indomethacin (1 µM, Sigma-Aldrich) to inhibit the production of prostaglandins. The baths were aerated with 95% O₂ : 5% CO₂ and maintained at 35-37 °C. Resting tension of 1g was applied to the tissues and isometric responses were recorded with a force transducer and displayed using the Chart program (ADInstruments, Sydney, Australia). Tissues were allowed to equilibrate for at least 10 min and frequency response curves to electrical field stimulation (EFS, 2, 5, and 20 Hz, 0.5 msec pulse width, 100 pulses, 60 V) were obtained before the addition of drugs. Carbachol (0.1, 1, and 10 µM; Sigma-Aldrich) was also tested cumulatively. Tissues were

also preincubated with tetrodotoxin (1 μ M; Alomone Labs, Jerusalem, Israel) for 10 min prior to EFS. All isometric tension recording data were analysed using Chart v7.

Imaging

Preparations were analysed by confocal microscopy on a Zeiss Meta or Zeiss Pascal confocal laser scanning system. Fluorophores were visualized using a 488 nm excitation filter with a 522/535 nm emission filter for Alexa 488, and 568 nm excitation with 605/632 nm emission filters for Texas Red. Z-series images and single 1024 x 1024 pixel images (optical sections of 0.5 μ m nominal thickness) were captured.

Statistical analysis

Data were evaluated by two-way analysis of variance (ANOVA) followed by Tukey post-hoc test for multiple comparisons between groups. Analyses were performed using Graph Pad Prism (Graph Pad Software Inc., San Diego, CA). All data are represented as mean $\pm$ standard error of the mean (SEM). Differences were considered statistically significant at $P < 0.05$.

Results

General observations

During the period of occlusion, the intestinal region that was deprived of blood flow became purple. After blood flow was restored, the tissue resumed its normal pink colour. The animals recovered from anaesthesia uneventfully, and once they were awake, the mice were active and ate normally. The regions subjected to ischemia were reddened in samples taken at 3 and 24 hr, whereas the remainder of the intestine was macroscopically normal.

Histological observations

3hr reperfusion

The histological studies showed that damage was more severe in nNOS^{-/-} compared to nNOS^{+/+} mice (Fig. 1).

In nNOS^{-/-} mice, the ends of the villi were missing and there was active bleeding from the majority of villi into the lumen (Fig 1c). Tissue debris was found in the lumen. The bases of the glands were intact and Paneth cell granules were very prominent. The circular muscle

contained numerous severely damaged muscle cells (Fig 1f). The surface membranes of the cells could be recognised, but the interiors of the cells were almost empty, except for small aggregates of residual cytoplasm that were almost always centrally located (Fig 1f). Nuclear profiles were seen in some of these clear cells. The severe damage with clear cytoplasm was in the outer component of the circular muscle layer. The inner circular muscle layer, which contains specialised smooth muscle cells (Gabella 1974), was very much less affected and cells with clear cytoplasm were not seen in this layer. Muscle cells with clear cytoplasm were also seen in the longitudinal layer, but these were fewer than in the circular layer. The myenteric ganglia had a foamy appearance of the neuropil. The clear spaces in the neuropil were commonly larger and more prominent than sometimes occurs if fixation is imperfect. The majority of nerve cells were affected and showed different features of nerve cell damage including foamy cytoplasm, irregular shapes, nuclei displaced to the edges of cells and pyknotic nuclei. Damage to submucosal neurons was less obvious but some were swollen. There were also eosinophils and neutrophils at the level of the myenteric ganglia, whereas in control tissues there are none associated with myenteric ganglia.

In nNOS^{+/+} mice the ends of the villi were lost, but the prominent micro-hemorrhages were not seen. There were some accumulations of blood cells in the surviving mucosa. The stumps of the villi were commonly sealed off with cuboidal or low columnar epithelium, indicative of mucosal repair (Fig 1b). Muscle damage was less than in nNOS^{-/-} mice. The number of muscle cells with clear cytoplasm in the circular muscle layer was much fewer, but the small amount of damage in the longitudinal muscle was similar to that in the nNOS^{-/-} (Fig 1). There was little evidence of myenteric neuron damage. There was some foaminess of the neuropil, but this was less obvious than in the nNOS^{-/-} mice. There was also slight loss of cytoplasmic integrity in submucosal neurons. Neutrophils and eosinophils seen in histological sections were few at the level of the myenteric plexus. Neutrophil numbers were quantified using the myeloperoxidase histochemical technique (see below).

24hr reperfusion

In nNOS^{-/-} mice, the mucosal surface epithelium was restored, although the villous tips were sometimes irregular. There was swelling of the central lacteals and of the surrounding lamina propria that applied to all or nearly all villi. The villi were shorter than normal and there were some trapped hemorrhagic foci in the villi. The residual circular muscle was very thin, generally only 2-3 cells thick (Fig 1h). It is possible that this is the surviving internal circular muscle. The longitudinal muscle contained very few cells that could be recognised

as smooth muscle cells (Fig 1h). It contained numerous nucleated cells with very little cytoplasmic staining. These appear to be a mixture of fibroblasts, lymphocytes and other immune cells. The myenteric nerve cells that survived at 24hr had normal nuclei and a clearer than normal cytoplasm.

In nNOS^{+/+} mice, villi had regrown, were covered in epithelium and were longer than in nNOS^{-/-} mice, but there was some distortion at the tips. There were few micro-hemorrhages. The muscle layers were thicker (especially the CM) than in the nNOS^{-/-} mice and were relatively intact (Fig 1g). Enteric neurons had normal nuclei, but some had a clearer than usual cytoplasm. There were also some lymphocytes at the serosal surface.

Neutrophil Infiltration

Neutrophils were revealed by the MPO reaction and were counted in three compartments: (1) the longitudinal muscle plus the myenteric plexus; (2) the circular muscle; (3) the submucosal plexus plus the base of the mucosa. Neutrophils were not counted in the villi because of the loss of this part of the ileal wall at 3 hr (Fig 1). In absolute control animals, no neutrophils were found attached to the serosa, in the longitudinal muscle, associated with myenteric ganglia, or in the circular muscle. In the regions subjected to I/R, numerous neutrophils were associated with the longitudinal and circular muscle at 24 hr (Fig 2). The numbers of neutrophils were significantly higher in nNOS^{-/-} compared to nNOS^{+/+} mice ($P<0.05$). These occurred at the serosal surface, through the thickness of the muscle layer, and close to the myenteric ganglia (Fig 2). No indication of preferential targeting of the ganglia or muscle was detected. Very small numbers of neutrophils were also found in the longitudinal and circular muscle layers of non-occluded segments of ileum, 5 cm proximal to the site subjected to I/R injury, and in segments of ileum from sham-operated animals (Fig 3). There was also neutrophil infiltration in the submucosa and mucosa after surgery, in the regions subjected to I/R, oral to this region and in the shams. This infiltration appears to be distributed randomly in the mucosa and submucosa compartment (Fig 2, 3).

α -Smooth muscle actin immunohistochemistry

α -Smooth muscle actin (SMA) immunoreactivity is a specific marker of differentiated smooth muscle cells in the gut (Knowles et al. 2009). In absolute control and sham-operated animals, SMA immunoreactive smooth muscle cells were well-defined and organised in regular bundles (Fig 4). The majority of cell profiles in the muscle layers were SMA immunoreactive. Following 3 and 24 hr reperfusion, significantly fewer of the cells in the

external muscle layers of both nNOS^{-/-} and nNOS^{+/+} mice had SMA immunoreactivity, and immunoreactivity was commonly confined to a peri-nuclear ring (Fig 4). To provide quantitative data, we counted the number of SMA immunoreactive cells per millimetre length of intestine (Fig 5). There were no significant differences in the numbers of smooth muscle cells with SMA immunoreactivity between absolute control, sham, and non-occluded regions. In the occluded regions, there was a significant reduction in the number of SMA immunoreactive cells in nNOS^{-/-} and nNOS^{+/+} mice compared to sham-operated animals following 3 hr reperfusion ($P<0.001$) (Fig 5). At 24 hr of reperfusion, there was a further reduction in the number of SMA immunoreactive cells in nNOS^{-/-} and nNOS^{+/+} mice ($P<0.001$). Furthermore, the number of SMA immunoreactive cells was significantly fewer in nNOS^{-/-} compared to nNOS^{+/+} at 3 and 24 hr of reperfusion ($P<0.001$) (Fig 5).

Immunoreactivity for nNOS and VIP

In the small intestine from normal mice, nNOS is co-localised with VIP in the terminals of enteric inhibitory neurons that innervate the circular muscle. In the nNOS^{-/-} mice, nNOS can no longer be detected. However, the innervation of the circular muscle by VIP immunoreactive nerve fibres was the same in the intestines from nNOS^{+/+} and nNOS^{-/-} mice (Fig 6), indicating that knockout of nNOS did not detectably change the pattern of innervation of the muscle.

Contractions of the longitudinal muscle

The muscle of the small intestine from control mice exhibited regular rhythmic tension changes (Fig 7). The contractions were of smaller amplitude in segments of intestine from sham operated mice (Fig. 7) and were of even lower amplitude in segments from nNOS^{-/-} and nNOS^{+/+} mice that were subjected to I/R. To quantify changes in basal contractile activity of the longitudinal muscle, we determined the motility index (frequency of contractions x average contraction amplitude) from occluded, non-occluded, and sham-operated regions (Fig 7). There were no significant differences in the motility index in absolute control mice compared to sham non-occluded regions (Fig 7). Following 3 hr of reperfusion, there was a significant reduction in the motility index in the occluded and non-occluded regions subjected to I/R compared to absolute control animals ($P<0.001$) (Fig 7). This reduction was even greater and contractions were almost abolished in the occluded regions following 24 hr reperfusion ($P<0.001$) (Fig 5). Moreover, there was a significant reduction in the sham-occluded regions when compared to absolute control samples ($P<0.001$) (Fig 7).

In small intestine from absolute control, occluded, non-occluded, and sham-operated animals, electrical field stimulation (EFS, 2, 5, 10Hz) induced frequency-dependent transient longitudinal muscle contractions (Fig 8). These contractions to EFS were abolished by TTX (1 μ M) indicating that they were of neuronal origin in all regions. For all of the frequencies tested, there were no significant differences in the amplitudes of contractions between absolute control and sham-operated mice. There were also no significant differences in any of the groups when stimulated at 2Hz (Fig 8b). At 3 hr of reperfusion, there was a significant reduction in the amplitude of contraction of preparations from both nNOS^{-/-} and nNOS^{+/-} mice when stimulated at 5 Hz compared to sham-operated mice ($P<0.05$). Following 24 hr reperfusion, the amplitudes of contractions were significantly lower in tissue from nNOS^{-/-} and nNOS^{+/-} mice when stimulated at 5Hz and 20Hz when compared to sham ($P<0.05$). Additionally, the occluded regions from nNOS^{-/-} mice were significantly less responsive compared to nNOS^{+/-} mice following 24 hr reperfusion ($P<0.05$).

The muscarinic receptor agonist carbachol (0.1, 1, 10 μ M) induced concentration-dependent sustained contractions in all samples. There were no significant differences in any of the groups when 0.1 μ M of carbachol was added (Fig 9). There were no significant differences in the amplitude of contractions in response to carbachol (1 and 10 μ M) between absolute control, non-occluded, and sham-operated mice. In the occluded regions, the amplitude was significantly lower in tissue from nNOS^{-/-} and nNOS^{+/-} mice following 3 and 24hr reperfusion when compared to sham-operated mice ($P<0.05$). In addition, the occluded regions from nNOS^{-/-} mice was significantly less responsive compared to nNOS^{+/-} mice following 24 hr reperfusion ($P<0.05$).

Discussion

The present study revealed that deletion of nNOS resulted in markedly greater I/R-induced damage to the small intestine than occurs in normal animals, suggesting that NO produced by nNOS plays a protective role following intestinal I/R. In nNOS^{-/-} mice, there was severe damage to the external muscle, increased neutrophil infiltration, fewer α -smooth muscle actin immunoreactive muscle cells, lowered contractile activity and reduced muscle responsiveness to nerve stimulation or a muscarinic agonist compared to nNOS^{+/-} mice.

Other animal experiments also indicate that NO has protective roles in the intestine, consistent with the present result with knockout of nNOS. Inhibition of all NOS isoforms,

using the broad spectrum NOS inhibitor, L-NAME, in the drinking water, caused ileitis (Miller et al. 1994), suggesting the net effect of NO in the intestine is protective, even in animals that are not artificially challenged. However, the intestine is always under a low level of challenge; there is always foreign material in the lumen, large numbers of immune cells are in the mucosa, and inducible NOS is constitutively present (Hoffman et al. 1997; Furness et al. 1999; McCafferty et al. 1999). In animals challenged by gastrointestinal inflammation, inhibition of NOS, with L-NNA or L-NAME, increased alcohol-induced gastric damage (Masuda et al. 1995) and exacerbated trinitrobenzenesulphonate-induced colitis in rats (Pfeiffer and Qiu 1995).

NO donors reduce the intestinal permeability increase that occurs after I/R (Payne and Kubes 1993). Consistent with this result, injection of L-NAME prior to I/R increased dextran leakage across the intestinal mucosa, compared to I/R alone (Luo et al. 2001). These studies did not examine mucosal structure. However, our histological investigation indicates that there is greater mucosal damage in nNOS^{-/-} mice, suggesting that the results of Payne et al. and Luo et al. can be explained by the influence of NO on the physical breakdown of the mucosal barrier. These results suggest a role for the nNOS that has been reported to be expressed by mucosal epithelial cells in the intestine (Torihashi et al. 1996) in mucosal protection.

Results obtained in the intestine subjected to I/R by Taha et al. (2010), who used pharmacological block of NOS, are in apparent contrast to our data. They reported that the reduction in contractions of the isolated rabbit jejunum, caused by ischemia for 1 hr plus reperfusion for 2 hr before the jejunum was removed, was less after blocking all NOS isoforms (whereas we observed an increased deficit with nNOS knockout). They gave L-NAME prior to applying the vascular clamp and during reperfusion. Contractions were elicited by transmural stimulation or KCl. This suggests that blocking all NOS isoforms (Taha et al. 2010) has a different outcome compared to just inactivating nNOS (present study). The results of our study and that of Taha et al. (2010) supports the hypothesis that nNOS is involved in normal physiological control in the intestine, and that its role is protective, whereas iNOS is capable of producing large amounts of NO which causes tissue damage (Kubes and McCafferty 2000; Kobayashi 2010). Also consistent with this hypothesis, Beck et al. (2004) found that knockout of nNOS caused a more severe dextran sodium sulphate-induced colitis, whereas dextran sodium sulphate was less damaging in iNOS knockouts.

Cytoprotective roles of NO

A number of mechanisms through which NO could have protective actions in the intestine are feasible, including inhibition of neutrophil extravasation, inhibition of vascular permeability increases, vasodilation increasing local blood flow and inhibition of cytokine production or action. Kubes et al. (1991) reported that NOS inhibitors increased leukocyte adhesion to the vascular endothelium and enhanced extravasation. Essentially similar results were found by Kurose et al. (1993) who used direct observation to view leukocyte adhesion and extravasation from mesenteric vessels *in vivo*. Moreover, NO and NO-releasing compounds inhibit neutrophil adhesion to endothelial cells (Grisham et al. 1998; Kosonen et al. 1999). During intestinal reperfusion, neutrophils rapidly infiltrate the external musculature and secrete molecules, including myeloperoxidase and elastase, that can damage the tissue (Massberg and Messmer 1998; Hierholzer et al. 1999). As we have confirmed in mice (Pontell et al. 2011), I/R injury to human tissue triggers neutrophil invasion of the external muscle and it also activates macrophages (Türler et al. 2002). In the current study, we found that the infiltration of neutrophils into the muscle layers of the region that was subjected to ischemia was greater in nNOS^{-/-} mice compared to nNOS^{+/+} mice.

There is also evidence that NO produced by nNOS suppresses NF-κB activation and subsequently reduces iNOS expression in cultured astrocytes (Togashi et al. 1997). In the intestine, inhibition of nNOS with the selective inhibitor 7-nitroindazole, increased levels of NF-κB (Qu et al. 2001). It is also possible that NO also has a cytoprotective role in the intestine during and immediately after ischemia by promoting vasodilation (Pique et al. 1989).

Ileus

It is common after almost all abdominal surgery that there is a period of intestinal hypomotility, referred to as post-operative ileus (Bauer and Boeckxstaens 2004; Lubbers et al. 2010). Ileus generally lasts 1 - 5 days, but it can persist for several weeks in some patients (Livingston and Passaro 1990; Lubbers et al. 2010). An inflammatory cascade is initiated when the intestine is handled, most notably in the muscular layers of the intestine (Kalff et al. 1998a; Kalff et al. 1999). This prominent inflammation is thought to initiate the hypomotility in the manipulated areas and can also lead to generalised stasis in the gastrointestinal tract (Lubbers et al. 2010). In the present study we observed a depression of motility in sham operated animals and we found neutrophils in the external muscle, although there are none in control. Thus we attribute the hypomotility in sham animals to post-operative ileus. This

indicates that after I/R in the current study, there is both a post-operative ileus and an effect of I/R on motility. The muscle damage observed after I/R was not observed in sham operated rats.

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FIGURES DESCRIPTIONS

Fig 1 Histological changes following ischemia/reperfusion injury in $nNOS^{-/-}$ and $nNOS^{+/+}$ mice. (a) In sham-operated animals, the villi, circular and longitudinal muscle had normal structure. (b) After 3 hr reperfusion, the stumps of the villi were commonly sealed off with cuboidal epithelium in $nNOS^{+/+}$ mice. (c) In $nNOS^{-/-}$ mice, the ends of the villi were still missing and there was active bleeding into the lumen following 3hr reperfusion. Tissue debris can be seen in the lumen. (d) In sham-operated animals, the circular (CM) and longitudinal muscle (LM) exhibited regular appearance. (e) After 3 hr reperfusion, the CM was relatively intact in $nNOS^{+/+}$ mice. (f) In $nNOS^{-/-}$ mice, the CM was severely affected and contained numerous damaged CM cells. (g) After 24 hr reperfusion, the LM was relatively intact in $nNOS^{+/+}$ mice. (h) There was severe LM degeneration in $nNOS^{-/-}$ mice following 24 hr reperfusion and this layer was replaced by numerous nucleated cells which appeared to be a mixture of immune cells and fibroblasts. Scale bars: a = 100 μ M; b-h = 50 μ M

Fig 2 Neutrophils revealed by myeloperoxidase histochemistry (dark brown cells). (a) & (d) In sham-operated mice, neutrophils were confined to the mucosa and submucosa, very few or none are present in the circular or longitudinal muscle but they occurred adjacent to mucosal glands. (b) & (e) After 3 hr reperfusion, neutrophils were common in the mucosa from occluded and non-occluded regions of nNOS^{-/-} and nNOS^{+/+} mice. (c) & (f) After 24 hr reperfusion, neutrophils were common in both the mucosa and muscle of occluded regions in both nNOS^{-/-} and nNOS^{+/+} mice. However, there appears to be more neutrophils in nNOS^{-/-} mice. Scale bar = 50 μ M, applies to all panels

Fig 3 Numbers of neutrophils per millimetre length of intestine. Neutrophil numbers in the longitudinal (a) and circular muscle (b) layers following 24 hr reperfusion were significantly increased in the occluded regions when compared to absolute control and sham-operated animals ($P<0.001$). Neutrophil numbers were also significantly higher in nNOS^{-/-} compared to nNOS^{+/+} mice ($P<0.05$) in the occluded regions 24 hr following reperfusion in the longitudinal and circular layers. Neutrophil infiltration appears to be distributed randomly in the mucosa and submucosa (c) compartment.

Fig 4 α -Smooth muscle actin (SMA) immunoreactivity in nNOS KO and WT mice following ischemia/reperfusion injury. (a) & (d) In nNOS^{+/+} and nNOS^{-/-} sham-operated animals, SMA immunoreactive cells are well defined and organised. (b) & (e) Following 3 hr reperfusion, there were fewer SMA immunoreactive cells. In the longitudinal muscle many of the cells that were identified by DAPI staining had no SMA immunoreactivity. (c) & (f) SMA immunoreactivity was lost from many cells after 24 hr reperfusion and cells in the longitudinal muscle from nNOS^{-/-} animals had little cytoplasmic SMA. Green = SMA; Red = DAPI. Scale bars = 20 μ M

Fig 5 Number of α -Smooth muscle actin (SMA) immunoreactive cells per millimetre length of intestine. There were no significant differences in the number of SMA immunoreactive cells between absolute control, sham, and non-occluded regions ($P>0.05$). Following 3 and 24hr reperfusion, there was a significant reduction in the number of SMA immunoreactive cells in both nNOS^{-/-} and nNOS^{+/+} mice ($P<0.001$). Furthermore, the number of SMA immunoreactive cells was significantly fewer in nNOS^{-/-} compared to nNOS^{+/+} mice at both time points ($P<0.001$).

Fig 6 VIP immunoreactivity in nNOS^{+/+} and nNOS^{-/-} mice. The innervation of the circular muscle by VIP immunoreactive nerve fibres was the same in the intestines from nNOS^{+/+} (a) and nNOS^{-/-} (b), indicating that knock out of nNOS did not detectably change the pattern of innervation of the muscle. Scale bar = 20 μ M, applies to both panels

Fig 7 Examples of contractile activity and motility index in absolute control, sham, occluded, and non-occluded regions of nNOS^{-/-} and nNOS^{+/+} mice. (a) Typical tracings of longitudinal muscle contractions from the mouse distal ileum showing the basal spontaneous activity. (b) Quantitative data of the changes in the motility index following I/R. There was a significant reduction in the motility index in the occluded and non-occluded regions of nNOS^{-/-} and nNOS^{+/+} mice compared to absolute control animals following 3 hr reperfusion ($P < 0.001$). This reduction was greater in the occluded regions subjected to I/R after 24 hr reperfusion ($P < 0.001$). The sham-occluded regions were also significantly affected ($P < 0.001$).

Fig 8 Responses to electrical field stimulation (EFS; 2, 5, 20Hz) following I/R. (a)

Typical tracings showing the contractions elicited in response to EFS at 5 Hz. (b) There were no significant differences in the amplitudes of contraction following 2Hz stimulation. At 5 (c) and 20Hz (d) the amplitudes of contractions were significantly lower in the occluded regions of nNOS^{-/-} and nNOS^{+/+} compared to sham-operated mice following 24hr reperfusion ($P<0.05$). Furthermore, the occluded regions from nNOS^{-/-} mice were significantly less responsive compared to nNOS^{+/+} animals following 24 hr reperfusion ($P<0.05$).

Fig 9 Responses to carbachol (0.1, 1, 10 μM) following I/R. (a) There were no significant differences in the amplitudes of contraction following the addition of 0.1 μM carbachol in any of the groups ($P>0.05$). At 1 (c) and 10 μM (d) amplitude of contractions were significantly lower in occluded regions from $n\text{NOS}^{-/-}$ and $n\text{NOS}^{+/+}$ compared to shams 24hr following reperfusion ($P<0.01$). In addition, the occluded regions from $n\text{NOS}^{-/-}$ mice were significantly less responsive compared to $n\text{NOS}^{+/+}$ mice following 24 hr reperfusion ($P<0.05$).