Acute paraquat exposure impairs colonic motility by selectively attenuating nitrergic signalling in the mouse☆

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A B S T R A C T

Paraquat, a common herbicide, is responsible for large numbers of deaths worldwide through both deliberate and accidental ingestion. Previous studies have eluded that the bioavailability of paraquat increases substantially with increasing dose and that these changes may in part be due to the effects that these high concentrations have on the gastrointestinal tract (GI tract). To date, the actions of acute, high concentrations (20 mM for 60 min) of paraquat on the GI tract, particularly the colon which is a major site of paraquat absorption, are unknown. This study examined the effects of acute paraquat administration on colonic motility in the C57BL/6 mouse. Acute paraquat exposure decreased colonic motility and the amplitude of colonic migrating motor complexes (CMMCs), which are major motor patterns involved in faecal pellet propulsion. In isolated segments of distal colon, paraquat increased resting tension and markedly attenuated electrical field stimulation-evoked relaxations. Pharmacological dissection of paraquat's mechanism of action on both the CMMCs and field stimulated tissue using the nitric oxide synthase inhibitor NG-nitro-L-arginine and direct measurement of NO release from the myenteric plexus, demonstrated that paraquat selectively attenuates nitrergic signalling pathways. These changes did not appear to be due to alterations in colonic oxidative stress, inflammation or complex 1 activity, but were most likely caused by paraquat's ability to act as a redox couple. In summary, these data demonstrate that acute paraquat exposure attenuates colonic transit. These changes may facilitate the absorption of paraquat into the circulation and so facilitate its toxicity.

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1. Introduction

Paraquat is a widely used herbicide responsible for large numbers of deaths worldwide. Paraquat bioavailability is normally low (Chui et al., 1988; Gawarammana and Buckley, 2011; Kan et al., 2010) but increases substantially with increasing doses, potentially due to effects on the colon which is a major site of paraquat absorption (Gawarammana and Buckley, 2011). As reduced motility may provide a mechanism to facilitate paraquat absorption and increase its bioavailability, we have chosen to examine the effects of paraquat on colonic motility.

Motility involves coordinated muscle contractions/relaxations. Contraction is driven by activity in excitatory motor neurons that release acetylcholine and tachykinins (Gamage et al., 2013; Wade and Cowen, 2004). Relaxation involves the activation of inhibitory motor neurons which release a combination of nitric oxide, a purine (ATP or β-NAD) and vasoactive intestinal peptide (Akbulut et al., 2015; Cowen, 2000;

Patel et al., 2014; Thrasivoulou et al., 2006). Changes in the balance between contraction and relaxation can lead to an impairment of motility.

Paraquat can alter cellular and organ function in a variety of ways. It is a pro-oxidant that generates the superoxide free radical (Day et al., 1999) and has been used to induce oxidative stress in a wide range of tissues/cell types to mimic disease (Bove and Perier, 2012; Djukic et al., 2012; Drechsel and Patel, 2008; McCormack et al., 2005; Miller et al., 2009; Samai et al., 2008) and to examine the role of oxidative stress in the natural ageing process (Jung et al., 2009; Salmon et al., 2009; Van Raamsdonk and Hekimi, 2009). Paraquat also reduces the activity of protein complexes of the respiratory chain (Cocheme and Murphy, 2008; Gomez et al., 2007; Rodriguez-Rocha et al., 2013) and can induce inflammation (Aires et al., 2013; Ajjuri and O'Donnell, 2013; Bove and Perier, 2012).

This study examined the effect of paraquat on colonic motility and its mechanism of action.

2. Materials and methods

2.1. Animals

All procedures were carried out according to U.K. Animals (Scientific Act), 1986 and associated guidelines and were approved by the

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University of Brighton Ethics Committee. Male C57BL/6J mice were obtained from Harlan UK at 8 weeks of age and housed in groups of 3–4 until required. Animals were maintained at 19.0 ± 1 °C, 55% humidity and fed on a maintenance diet (RM1 (E) 801,002 chow, Special Diet Services) and had free access to water. The animals were kept on a 12 hour light/dark cycle and studied at 3–4 months of age. Mice were killed prior to experimentation by CO_2 (100%) asphyxiation, followed by cervical dislocation.

2.2. Pellet motility assays

The whole colon was harvested and placed in ice cold oxygenated (95% O_2 and 5% CO_2) Krebs buffer solution, pH 7.4 containing (117 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM NaH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose). The mesentery was trimmed using fine scissors and the whole colon was then loosely pinned in a Sylgard-lined flow bath, allowing a lateral movement of approximately 0.5 cm about the mid-line and perfused with oxygenated Krebs buffer solution at 37 ± 1 °C at a flow rate of 8 ml min^{-1}. A small (2 mm) incision was made in both ends of the colon and the openings pinned flat to facilitate pellet insertion and its expulsion at the distal end. If spontaneous evacuation was not achieved, the faecal pellets were removed from the isolated colon after 30 min, by gently flushing the lumen of the colon with warmed Krebs buffer solution. The colon was then left to stabilize for 15 min, prior to recordings of pellet motility. Measurements of motility were carried out using a 2 mm diameter epoxy-coated artificial faecal pellet. The artificial faecal pellet was inserted 3–4 mm into the proximal end of the bowel using a fire-polished glass capillary and the movement of the pellet was monitored using a video camera. Pellet motility was tracked using Ethovision tracking software. Following a successful trial, the experiment was repeated two further times and the average response utilized for statistical analysis. Tissues were then perfused with 20 mM paraquat for 60 min and pellet motility assayed for a further three trials. The maximum time that any single trial was conducted was 45 min. The total transit time of the artificial faecal pellet was recorded along with the distance and the average velocity of the pellet determined (Patel et al., 2014).

2.3. Measurement of colonic migrating motor complexes (CMMCs)

Briefly the whole colon was placed in a Sylgard-lined recording chamber and a thin metal rod (1 mm diameter) placed through the lumen and secured at each end to the Sylgard. Recordings of circular muscle contractions were made at two locations along the whole isolated colon, one at the proximal end and one at the distal end. Fine suture silk was tied through the muscle layers at each location and connected to two separate isometric force transducers. The muscle was placed initially under a low level of tension 4 mN and then tension increased over the next 40 min until a final tension of 6 mN was reached. The signal from each force transducer then passed to a preamplifier and ADI PowerLab before being stored on computer using Chart software. The tissue was perfused for 60 min prior to recording with either normal Krebs buffer solution or Krebs buffer solution containing 20 mM paraquat. Post this period, recordings of spontaneous CMMCs were made for 60 min before the addition of 100 μM NG-nitro-L-arginine (nitric oxide synthase inhibitor). The bath was allowed to equilibrate for 30 min with the NG-nitro-L-arginine before spontaneous CMMCs were again recorded for 60 min.

2.4. Electrical field stimulated distal colon segments

The whole colon was removed and 2 cm sections of distal colon were hung vertically in an organ bath containing Krebs buffer solution. Distal colon segments were chosen as their pharmacology in mice is far better understood than the proximal colon. Tissues were then incubated for 60 min in either control Krebs buffer or Krebs buffer containing 20 mM paraquat. At the end of this period the tissue was washed 4 times and 10 μM of guanethidine was added to the bath. The tissue was then dosed up with 100 μM acetylcholine for 1 min every 10 min, until successive applications yielded a consistent response. Following this a frequency response curve was generated by passing current pulses across the tissue (40 V, 0.3 ms pulse duration, 0.1–30 Hz). Tissues were stimulated for 30 s every 5 min. The tissues were then washed and the frequency response curves were repeated in the presence of either 100 μM NG-nitro-L-arginine or 1 μM scopolamine (muscarinic antagonist) + 1 μM GR159897 (NK_2 receptor antagonist) to block contractile pathways.

Responses to EFS can be obtained through activation of neurons within the plexi or directly through activation of the smooth muscle. To ensure that our protocol was selectively activating neurons 400 nM tetrodotoxin was added to the tissue to block neuronal voltage-gated Na^+ channels and the tissue again was stimulated at 10 Hz.

2.5. Detection of NO release from the myenteric plexus

Methods for the detection of NO release from the myenteric plexus have been described previously (MacEachern et al., 2011; Patel et al., 2010). 10 μM of veratridine (Na^+ channel activator) was used to evoke NO release to mimic the effects of electrical field stimulation.

For the following assays freshly isolated distal colonic segments were placed in either Krebs buffer solution or Krebs buffer solution containing 20 mM paraquat for 60 min. Following the incubation the tissues were washed and the colon was bisected along the mesenteric border to expose the mucosal tissue, which was then scraped away. The mucosa and remaining muscle layers were then stored separately.

2.6. Malondialdehyde assay

Malondialdehyde formation was utilized to quantify levels of lipid peroxidation in the tissue samples and measured as thiobarbituric acid-reactive material. Tissues were homogenized (100 mg ml^{-1}) in 1.15% KCl buffer. 200 μl of the homogenates was then added to a reaction mixture consisting of 1.5 ml 0.8% thiobarbituric acid, 200 μl 8.1% sodium dodecyl sulphate, 1.5 ml 20% acetic acid (pH 3.5) and 600 μl distilled H_2O. The mixture was then heated at 90 °C for 45 min. After cooling to room temperature, the samples were cleared by centrifugation (10,000 g, 10 min) and their absorbance was measured at 532 nm, using 1, 1, 3, 3-tetramethoxypropane as an external standard. The level of lipid peroxides was expressed as nmol MDA/mg protein (Bradford assay).

2.7. Western blot

The mucosa and remaining muscle layers were separately snap frozen in liquid N_2 for storage. Tissue was placed on ice in lysis buffer (10 mM HEPES, 150 mM NaCl, 1 mM EDTA, 0.2% Nonidet P40, protease inhibitor cocktail P8340, Sigma-Aldrich Inc.) and manually lysed. Lysates were centrifuged for 10 min, at 4 °C at 700 $\times g$ to pellet debris and nuclear components. Supernatants were removed and their protein content was assessed using Quick Start Bradford Dye reagent (Bio-Rad) 26. 15 μg of protein from each sample was combined with an equal volume of 2× Laemmli loading buffer (S3401, Sigma-Aldrich), separated on a 10% SDS-PAGE gel using the Mini-protean II electrophoresis cell (Bio-Rad) and transferred to Immobilon polyvinylidene fluoride (PVDF) membrane using the Trans-Blot® wet transfer (Bio-Rad). Membranes were blocked with 5% milk in PBS-Tween 20 (0.2%) for 3 h and then incubated overnight at 4 °C with either a rabbit anti-TNFα antibody (1:5000; Millipore) or a rabbit anti-complex 1 antibody (1:5000; Aviva systems) or a mouse monoclonal anti-actin antibody (1:5000; Santa Cruz) diluted in milk-PBS-Tween 20. Membranes were washed five times in PBS-Tween 20 and then incubated with goat anti-rabbit HRP-conjugated secondary antibody at 1:2000 (SC-2005, Santa Cruz