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Effects of infusing nitric oxide donors and inhibitors on plasma metabolites, muscle lactate production and meat quality in lambs fed a high quality roughage-based diet

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ABSTRACT

As nitric oxide (NO) is postulated to be a mediator of the effects of pre-slaughter stress on meat quality the aims of this experiment were to investigate the effects of modulating NO pharmacologically on meat quality of sedentary lambs. As pharmacological NO donors are prohibitively expensive to use in the lamb model L-Arginine, the substrate for NO synthase (NOS) was infused into lambs and increased NO production by ~30%. In a 2 × 2 factorial design we infused either L-Arginine (500 mg/kg) or the NOS inhibitor L-N^G nitroarginine methyl ester hydrochloride (L-NAME, 30 mg/kg) 190 min pre-slaughter and investigated meat quality in the *Longissimus thoracis lumborum* (LTL) or *Semimembranosus* (SM). The principal outcome of the experiment was that L-NAME inhibited proteolysis and reduced tenderness in the SM. These data indicate that events pre-slaughter that affect NO synthesis can influence meat tenderness, potentially via altered muscle metabolism or modulation of proteolytic enzymes.

1. Introduction

- Nitric oxide (NO) is an important regulator of skeletal muscle homeostasis where it regulates physiological pathways that are important to the development of meat quality. For example, NO is involved in the regulation of force production, blood flow, glucose metabolism, calcium homeostasis, and proteolysis (Balon & Nadler, 1985; Clark et al., 2003; Koh & Tidball, 2000; Zhang, Kraus, & Truskey, 2004). As skeletal muscle NOS activity is increased by exercise in humans (Jungersten, Ambring, Wall, & Wennmalm, 1985; Roberts, Barnard, Jasman, & Balon, 1999), it has been postulated that NO may be a mediator of the effects of pre-slaughter stress on meat quality (Cottrell, McDonagh, Dunshea, & Warner, 2008; Warner, Dunshea, Ponnampalam, & Cottrell, 2005). This was demonstrated in an experiment where stress, in the form of a short bout of imposed exercise, increased NO synthesis in lambs, as indicated by increased plasma nitrate and nitrite (NO_x) concentrations (Cottrell, Dunshea, Ponnampalam, & Warner, 2005). In a subsequent experiment, pharmacological inhibition of NOS with L-N^G nitroarginine methyl ester hydrochloride (L-NAME) in sedentary and exercising lambs 2 h pre-slaughter resulted in both exercise dependent and independent effects, including increased glycogenolysis and glycolysis. There was also a divergent effect on meat tenderness, such that the LTL became more tender overall and the SM become tougher in sedentary, but not in exercised lambs (Cottrell et al., 2008). As the changes in meat tenderness were independent of changes in ultimate pH, colour or water holding capacity, it was hypothesised that NO influenced meat tenderness by a proteolytic mechanism.

The use of NO to improve meat quality is not new as reduction of nitrates and nitrites to yield NO has traditionally been used for curing meat, increasing shelf life and improve colour (Cornforth, 1996; Walters & Taylor, 1964). However, it is only recently that a link between NO and fresh meat quality, specifically the tenderisation process, has been implicated. The first experiment to demonstrate an effect of NO on meat tenderness was demonstrated by Cook, Scott, and Devine (1998), who observed improvements in tenderness with pharmacological NO donors and reduced tenderness with L-NAME infused post-slaughter into strips of hot de-boned beef LTL. Using a similar approach, Li et al. (2014) observed that L-NAME infused post-slaughter increased pork LTL proteolysis

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and μ-calpain autolysis, which is a marker of increased calpain activity. However, the activity of NOS in vivo is tightly regulated, requiring O_2 and NADPH as substrates and FAD, FMN, BH_4, heme and calmodulin as cofactors (Reid, 1998) and are pH-dependent (Anderson & Meyer, 2000). Reductions in pH values below 7 uncouple NADPH oxidation by NOS, inhibiting the enzyme (Gorren, Schrammel, Schmidt, & Mayer, 1998). Therefore further experimentation is required to determine the association between endogenous pre-slaughter NO synthesis and meat quality. The interactions between NO synthesis, pre-slaughter stress and meat quality were investigated by Cottrell et al. (2008). However, pre-slaughter stress as used in this experiment activates many more physiological processes than NO synthesis alone. Targeted mechanistic studies into the action of NO in large animals are problematic due to the prohibitive costs associated with using pharmacological NO donors. Therefore, the aims of this study were two-fold, the first was to investigate if infusion L-Arginine, which is the substrate for NO synthesis could be used to stimulate endogenous NO synthesis. The second part was to investigate the subsequent effects of NO synthesis with L-Arginine and NOS inhibition on lamb meat quality.

2. Materials and methods

2.1. Animals and infusions

Two experiments were conducted, the first (Section 2.1.1) investigated the effects of different doses of L-Arginine infusion on NO synthesis as a basis for further investigations into meat quality (Section 2.1.2).

2.1.1. L-Arginine and NO synthesis dose response study

Five Border Leicester × Merino wethers weighing 50–55 kg were housed in individual metabolism crates and fed ad libitum, dispensed three-hourly with an auto-feeder and supplemented with approximately 100 g/day of lucerne hay as described by Cottrell, Warner, McDonagh, and Dunshea (2004). At about 24 h of the experiment, a catheter (12 G Dwell Cath, Sutherland Medical, Australia) was placed in the external jugular vein of restrained lambs and maintained with K_2EDTA (12.5 g K_2EDTA/L in 0.9% NaCl, Sigma Aldrich). An extension line was tunnelled underneath the lambs' wool, exiting in the middle of the back to minimise stress during sample collection. In a randomized cross-over design 0, 150 and 500 mg/kg bolus L-Arginine·HCl (Sigma) was administered as a bolus via the catheter. Each lamb received each dose of L-Arginine on three separate infusion days with 24 h wash out in between. Three milliliter blood samples were collected via the same catheter, placed on ice in a collection tube containing heparin and a separating gel (Sarstedt, Germany) then plasma was collected and frozen for later analysis after centrifugation at 4000 ×*g* for 10 min. Nitric oxide synthesis can be quantified by the concentration of plasma nitrite and nitrate (NO_x), which are stable degradation products of free NO (refer to Section 2.3.1 for analytical method). Increases in NO synthesis between L-Arginine doses were quantified by the area under the plasma concentration curve between 0 and 9 h (AUC), the maximal plasma NO_x concentration (C_{max}) and time to C_{max} (T_{max}). The AUC was calculated by the trapezoidal rule:

$$AUC = \Sigma_{0\text{-}9}\left(C_{n+1} - C_n\right)/2\right) \cdot T_2 - T_1\right).$$

2.1.2. L-Arginine and L-NAME meat quality study

In the second study, 40 s cross ((Merino × Border Leicester) × Poll Dorset) wether lambs weighing 43–45 kg were grazed on pasture with daily supplementation of lucerne hay and lamb pellets for two weeks. After two weeks in the paddock, animals were weighed, divided into four groups and housed in sheltered individual pens for two weeks and fed lucerne chaff ad libitum and lamb pellets (~250 g/day). The metabolisable energy and crude protein content of the lucerne chaff and pellets were 9 and 12 MJ ME/kg and 17.9 and 19%, respectively. At about 24 h prior to slaughter, lambs were catheterised as described in Section 2.1.1. Infusions began 190 min before slaughter and lambs were staggered in 20-minute intervals to facilitate the timing of sampling. Lambs were assigned to one of the four treatments (10 lambs per treatment) which was delivered in a 30 mL bolus: Control (saline), Arginine (500 mg L-Arginine·HCl/kg in saline, Sigma Aldrich); L-NAME (30 mg/kg in saline, Cayman Chemical Co.) or L-Arginine plus L-NAME (500 mg/kg L-Arginine plus 30 mg/kg L-NAME). The methyl group of L-NAME is rapidly hydrolysed in vivo to form L-N^G nitroarginine (L-NNA), which is considered the active form. The dose response of L-NAME and L-NNA in animals is equivalent (Traystman et al., 1995) and L-NAME is used due to its greater aqueous solubility. All procedures were approved by the Animal Ethics Committee (AEC No. 2461), Department of Environment and Primary Industries, Victoria.

2.2. Slaughter procedure and muscle sample collection

Lambs from Section 2.1.2 were restrained in a V-restrainer and electrically stunned with a dual point electrode placed to the head, exsanguinated and euthanised via cervical dislocation. Carcasses were trimmed according to the specifications of AUS-MEAT (Anonymous, 1992) and chilled at 2 °C and ~86% humidity overnight. Samples of muscle LTL and SM (~0.2 g) were collected at 15 min, 1, 3, and 24 h post-slaughter, trimmed of fat and connective tissue and snap frozen in liquid nitrogen for muscle glycogen and lactate determination. Muscle pH was measured after 15 min, 3, and 24 h post-slaughter in the LTL at the 13th thoracic vertebra and in the SM using a portable pH meter (Jenco Electric, CA) and polypropylene spear tip gel electrode (Ionode Pty Ltd, Australia).

Approximately 24 h post-slaughter, the LTL and SM were randomly selected from one side and removed from the carcass. Each muscle was halved and randomly allocated for immediate analysis (1 day post-slaughter) or vacuum packed and stored for analysis at day 3. The 1 day post-slaughter sample was split into samples for Warner–Bratzler shear force (WBSF) and cooking loss (>80g), sarcomere length (4 samples cut parallel with the fibres in 4 cm × 1 cm^2 strips, frozen in liquid nitrogen and stored at −20 °C), myofibrillar fragmentation index (MFI, 20 g, frozen at −20 °C) and surface colour (2.5 cm thick sample). The 3 day post-slaughter samples were weighed then vacuum packed in plastic bags and stored at 2 °C and after ageing, were removed for WBSF, purge, colour, cooking loss and MFI measurements.

2.3. Measurements and analytical procedure

2.3.1. Biochemical analyses

Plasma glucose and lactate were quantified from plasma using the glucose and lactate oxidase methods as per manufacturer's guidelines (Sigma Aldrich, MO). Plasma NO_x is a marker of free NO and was determined following the manufacturer's guidelines (Cayman Chemical Co., USA) as reported by Cottrell et al. (2004). Briefly, plasma was filtered using 10K MW cut-off centrifugal filters, then nitrite was colorometrically quantified after the reduction of nitrate to nitrite. Muscle glycogen concentration was determined from frozen (−20 °C) supernate of LTL and SM samples (0.2 g) that were homogenised in 30 mM cold HCl using a bead beater with 0.5 mm silica beads (Biospec Products Inc., USA) followed by centrifugation at 13,500 xg for 5 min. For assay the supernate was added to a buffer (1:10) containing amyloglucosidase (Roche Diagnostics, 1:200 in 40 mmol acetate) and incubated for 90 min at 37 °C to convert the glycogen to glucose equivalents, which were then quantified using a COBAS MIRA S autoanalyser (Roche, Switzerland) and Infinity™ glucose hexokinase kit (Thermo Trace, Australia). The concentrations were then used to calculate the glycogen levels in mmol/g of muscle tissue. Muscle lactate was determined from the supernate by measuring the stoichiometric consumption of NAD^+ when converting lactate to