Xanthine Oxidase Formation during Experimental Ischemia of the Equine Small Intestine

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ABSTRACT

We hypothesized that xanthine oxidase plays a role in the postischemic reperfusion injury in the equine small intestine. Under anesthesia, four horses and two ponies underwent ischemic strangulating obstructions of segments of the proximal jejunum, mid-jejunum and ileum. Prior to vascular occlusion, and at 1 h and 2 h of ischemia, full-thickness intestinal biopsies were collected for histopathological evaluation and for determination of combined xanthine dehydrogenase (XDH) plus xanthine oxidase (XO) activity, and XO activity alone. The level of XO activity was expressed in percentage according to the ratio of XO/(XDH + XO). We found a nearly threefold increase in the combined level of XDH plus XO activity from ileum to duodenum ($p < 0.04$). However, the preischemic level of % XO activity did not vary significantly ($p = 0.61$) between segments of jejuno-ileum. Likewise, no significant difference was noted between intestinal segments after ischemia. Therefore, the data from all intestinal segments were pooled for each time and analyzed using Wilcoxon's signed rank test (one-tailed). Compared to the pre-ischemic level of % XO activity (median 27%), the % XO activity increased after 1 h of ischemia (median 37.0%), reaching statistical significance ($p = 0.016$). There were no statistical differences between the preischemic % XO activity and the % XO activity in non-ischemic bowel at the end of the anesthetic period. During ischemia, % XO activity increased, which lends credence to the importance of xanthine oxidase in previously-documented reperfusion injury in the equine small intestine.

RÉSUMÉ

L'hypothèse voulant que la xanthine oxydase soit impliquée dans le processus ischémique lors d'accidents vasculaires impliquant le petit intestin du cheval a été émise. Une ischémie obstructive des segments proximaux et moyens de jéjunum ainsi que de l'iléon a été induite par strangulation chez quatre chevaux et quatre poneys. Avant la période ischémique et une et deux heures après, des biopsies intestinales ont permis d'évaluer le type de lésions ainsi que les niveaux de xanthine oxydase (XO) seule ou combinée à la xanthine déshydrogénase (XD). L'activité de la XO fut exprimée en pourcentage de la quantité totale de XO + XD. Les niveaux de XO + XD ont augmenté ($p < 0,04$) de trois fois de l'iléon au duodénum. Par contre, les taux pré- et postischémiques de la XO n'ont pas varié entre les segments intestinaux étudiés. Pour chaque période, lorsque les données pour tous les segments intestinaux furent analysées pour chaque temps par la méthode de Wilcoxon, une augmentation significative ($p = 0,016$) du taux de XO a été observée après une heure d'ischémie. Aucune différence du taux de XO n'a été décelée entre les niveaux pré et postischémiques à la fin de la période d'anesthésie. Les résultats de cette étude corroborent ceux rapportant une augmentation des taux d'activité de l'enzyme XO lors d'accidents circulatoires au niveau du petit intestin du cheval.

INTRODUCTION

Strangulating intestinal obstructions have been reported to account for 18% of horses presented to referral hospitals for treatment of abdominal pain (1). Strangulation obstruction of the small intestine has been associated with a case fatality rate of 70% (1). An important factor in intestinal viability is the type and severity of the vascular insult. To determine intestinal viability, many techniques, with varying accuracy, have been investigated (2–13). In 1980, the concept of reperfusion injury was proposed following investigation of the feline small intestine (14–15). This concept indicated that production of superoxide and hydroxyl radicals during the reperfusion phase may result in further cellular damage. This could partially explain the inaccuracy associated with intestinal viability determined intraoperatively. A proposed pathway for the formation of superoxide radicals included the transformation of xanthine dehydrogenase (XDH) to xanthine oxidase (XO) during the ischemic period. In horses, Horne *et al* (16) reported that more severe histological lesions were observed after 3 h of arteriovenous ischemia and 1 h of reperfusion compared to 4 h of arteriovenous ischemia (16). Thus, reperfusion injury of the equine small bowel has been observed experimentally.

The purpose of this investigation was to determine the level of total enzymatic activity of XDH plus XO,

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Submitted March 7, 1991.

and the level of XO activity in various segments of the nonischemic equine small intestine. This would lead to calculation of XDH activity. In addition, we wished to investigate whether conversion of xanthine dehydrogenase to xanthine oxidase occurred under arteriovenous ischemic conditions by determinating XO expressed as a percentage of total enzyme activity (XDH + XO). Confirmation of XO formation in ischemic equine small intestine is a prerequisite for future projects attempting to block XO formation.

MATERIALS AND METHODS

ANIMALS

Four mature (> 2 years) horses and two ponies (136–572 kg) were obtained and determined to be in good condition following a detailed, systematic, physical examination and complete hematological profile. All animals were donated to the large animal clinic and had been scheduled to be euthanized for reasons unrelated to the gastrointestinal tract. All procedures complied with institutional and federal animal care and welfare policies and guidelines and were approved by the local institutional animal care and use committee. The animals were housed in box stalls and fed a diet of hay and water.

ANESTHETIC PROCEDURES

Prior to surgery, the animals were fasted for 8 h. Anesthesia was induced with intravenous xylazine (1.1 mg/kg), diazepam (5–15 mg total dose), and ketamine (2.2 mg/kg). Following intubation, anesthesia was maintained by halothane and oxygen inhalation throughout the procedure. Halothane was used as it is the anesthetic agent most commonly used for anesthesia of surgical colic cases (17). The mean blood pressure, arterial PO2 and arterial PCO2 were monitored and maintained within normal limits (mean arterial pressure > 60 torr, arterial oxygen > 100 torr, arterial CO_2 < 60 torr). Isotonic and polyionic intravenous fluids and dobutamine (5–10 μg/kg/min to effect) were administered when necessary to maintain mean blood pressure. The average duration of anesthesia was 4 h.

SURGICAL PROCEDURE

The horses were placed in dorsal recumbency and prepared for surgery. A ventral midline celiotomy and a routine abdominal exploratory examination were performed. The small intestine was exteriorized and divided into proximal, middle and distal segments. Full-thickness biopsies were collected from each segment for xanthine dehydrogenase/oxidase assay and histopathology at the following periods: prior to occlusion, after 1 h and 2 h of ischemia in the ischemic segments and at the end of the ischemic period in a nonischemic (control) segment. The location of the biopsies was: the proximal jejunum — starting 30 cm distal to the duodenocolic ligament (proximal segment), mid-jejunum (middle segment), and ileum (distal segment). Following the collection of biopsies, a complete (venous, arterial and mural) vascular occlusion was created in all three segments. Complete vascular occlusion was achieved by ligation and transection of the appropriate mesenteric arteries and veins and by application of umbilical tape around the wall at each of the segments of bowel in which ischemia was created. Following collection of the biopsies, all horses were euthanized while under general anesthesia.

DATA COLLECTION

Histological examination — The intestinal biopsies were fixed immediately in 10% formaldehyde. All biopsies were trimmed to provide a full-thickness view of the intestine and to maximize the amount of mucosa available for the study. Trimmed specimens were embedded in paraffin, cut parallel to the villus crypt axis (5 μm sections) and stained with hematoxylin and eosin. The degree of mucosal damage was scored with the pathologist blind as to the identity of the animal and sample time. The samples were evaluated according to the classification scheme previously described by White *et al* (18) and summarized as: grade 0 — no abnormal findings; grade 1 — slight separation of epithelial cells from the lamina propria at the tip of the villus; grade 2 — loss of epithelial cells from the tip of the villus with minimal congestion and/or hemorrhage within the lamina propria; grade 3 — loss of epithelial cells such that one-third to one-half of the lamina propria of the villus was exposed with mild to moderate congestion and hemorrhage within the lamina propria; grade 4 — complete loss or separation of villus epithelium exposing the entire villus lamina propria, with severe lamina propria hemorrhage and sub-mucosal hemorrhage; grade 5 — loss of villus architecture and early necrosis of the crypt cells.

Xanthine dehydrogenase and oxidase determinations — Measurements of these enzymes were performed on crude tissue homogenates originating as mucosal portions of the full-thickness intestinal biopsies. Xanthine oxidase activity was measured using the dual spectrophotometric technique of Parks *et al* (19) and adapted to equine mucosa by Wilkins (20). Briefly, a 2 g portion of the small-intestinal mucosa was collected for determination of total XO plus XDH activity and XO activity alone. Following biopsy, the mucosa was separated from the seromuscular layer and frozen immediately in liquid nitrogen. The 2 g of the mucosa were homogenized in 20 mL of homogenizing buffer containing 50 mM potassium phosphate, 10 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride, and 0.1 mM ethylenediamine tetraacetic acid (EDTA), at pH 7.0 using a polytron. This crude tissue homogenate then was centrifuged at 110 *g* for 30 min at 4°C. The supernatant was collected and stored at −20°C for less than 24 h until the assay could be performed. The tissue pellet was discarded. All samples were assayed within 24 h of collection. The assay is based on a crude tissue homogenate spectrophotometric measurement of uric acid production. The reaction includes 50 μM xanthine, 100 μM EDTA, and 50 mM potassium phosphate, pH 7.8, leading to measurements of combined xanthine dehydrogenase plus xanthine oxidase activity. With the addition of nicotinamide adenine nucleotide (500 μM) xanthine oxidase activity is measured. Xanthine dehydrogenase activity is calculated by subtracting XO activity from the combined enzyme activity (XDH + XO). Production of uric acid was deter-

mined on a Beckman spectrophotometer at 295 nm over a 5 min period at 25°C. The supernatant was also assayed under the same condition but in the presence of 100 μM allopurinol, a specific inhibitor of XDH and XO. Enzymes activities were expressed in nanomoles/min and gram of wet issue (nmol min^{-1} g^{-1}) by the following formula:

$Absorption_{295}/min$ X (1.1 X 10^4 M^{-1} $ABS_{295})^{-1}$ × (cuvette volume in L/sample volume in mL) × (g tissue/homogenate volume + g tissue in mL)$^{-1}$

Where: 1 nmole urate/min = units and the millimolar extinction coefficient is 1.1 × $10^4 M^{-1}$

STATISTICAL ANALYSIS

In the preischemic sample, the combined level XDH + XO activity was compared using Friedman two-way analysis of variance with the intestinal segment and the horse as the variables. Likewise, the % XO activity prior to ischemia was compared using Friedman two-way analysis of variance with the intestinal segment and the horse as the variables. To determine whether conversion of XDH to XO occurred, the ratio of XO/(XDH + XO) was expressed as a percentage. Using these ratios, within intestinal segments, comparisons between 1 h ischemia, 2 h ischemia, or 2 h nonischemia with the preischemic sample were made using the one tail Wilcoxon signed rank test. The level of significance was set at $p < 0.05$ per overall comparison and using the Bonferroni adjustment, was set at $p < 0.017$ per individual comparisons. Since no significant difference ($p = 0.03$) was found between the ratios of XO/(XDH + XO) of each intestinal segment, the data from all intestinal segments within a sampling time were pooled (using the median value of all 3 segments) and compared as described above using the one tail Wilcoxon signed rank test.

RESULTS

Following vascular occlusion the bowel rapidly became cyanotic and, after 2.5 h of ischemia, was grossly thickened and gray in color. The adjacent bowel was grossly normal in appearance. Results of histopathological examination of the intestinal biopsies are shown in Table I.

TABLE I. Results of histopathological examination of the equine small intestines following one and two hours of complete ischemic strangulation obstruction

Sampling Time	Median histological grade (range)		
	Proximal small intestine	Middle small intestine	Distal small intestine
Preischemia	0 (0–0)	0 (0–0)	0 (0–0)
2 h anesthesia, No ischemia	0 (0–0)	0 (0–0)	0 (0–0)
1 h ischemia	2 (2–3)	2.5 (2–3)	2 (1–2)
2 h ischemia	3 (3–4)[a]	3 (3–3)	3 (3–4)

[a]One mislabeled sample was eliminated

TABLE II. Summary of xanthine dehydrogenase (XDH), xanthine oxidase (XO), and total (XDH + XO) activity in three segments of the equine small intestine

Horse no.	Enzyme activity nmol $min^{-1}g^{-1}$			%
	(XDH)	(XO)	(XDH + XO)[a]	XO/(XDH + XO)[b]
Proximal small intestine				
1	53.9	21.6	75.5	29
2	110.6	37.9	148.5	26
3	97.9	23.9	121.8	20
4	108.0	37.5	145.5	26
5	83.3	25.7	109.0	24
6	85.0	55.3	140.3	40
Median	91.1	31.6	131.1	26
Middle small intestine				
1	19.1	70.7	89.8	79
2	65.0	13.0	78.4	17
3	100.6	23.4	124.0	19
4	46.5	14.4	60.9	24
5	42.3	20.2	62.5	32
6	70.3	33.9	104.2	33
Median	55.7	21.8	84.1	28
Distal small intestine				
1	66.3	17.4	83.7	21
2	33.4	11.1	44.5	25
3	53.6	15.8	69.4	23
4	45.1	6.4	51.5	13
5	26.1	15.2	41.3	37
6	22.2	21.3	43.5	49
Median	32.3	15.5	48.0[a]	24

[a]Difference between intestinal segments is statistically different ($p = 0.04$)
[b]No statistical difference between intestinal segments ($p = 0.61$)

Results of total enzyme activity within each segment are shown in Table II. The level of total enzyme activity (XDH plus XO) was highest in the proximal small intestine (median 131.0 nmol min^{-1} g^{-1}) and decreased progressively in the middle (median 84.1 nmol min^{-1} g^{-1}) and distal small intestine (median 48.0 nmol min^{-1} g^{-1}) ($p = 0.04$). There were no significant difference between horses ($p = 0.50$).

The XO/(XDH + XO) ratio expressed as a percentage is shown in Table III. The pooled preischemic % XO activity (median 27%) increased after 1 h of ischemia (median 37%, $p = 0.016$). Again in the pooled data, there was no statistically significant increase in % XO activity associated with 2 h of ischemia or the experimental procedure ($p = 0.08$ and 0.06 respectively).

DISCUSSION

The gross and histological results of this study were consistent with ischemic strangulation obstruction in horses as reported by Sullins and Horne (8,16).

TABLE III. Xanthine oxidase activity expressed as percentage of total enzyme activity (XO + XDH) in horses subjected to two hours of complete vascular obstruction of three segments of the small intestine[a]

	Preischemia	1 h ischemia	2 h ischemia	2 h anesthesia
Proximal				
1	29	45	100	27
2	26	36	16	35
3	20	28	43	23
4	26	25	18	31
5	24	15	27	18
6	40	38	35	33
Median	26	32	31	29
Middle small intestine				
1	79	M	83	39
2	17	38	30	37
3	19	35	69	28
4	24	39	40	34
5	32	33	29	30
6	33	39	44	30
Median	28	38	42	32
Distal small intestine				
1	21	M	M	M
2	25	20	M	42
3	23	26	35	39
4	13	39	53	31
5	37	39	34	33
6	49	37	58	40
Median	24	37	44	39
Pooled Data				
Median	27	37[b]	41	32
Range	(20–33)	(28–45)	(23–92)	(28–37)

[a]There were no statistical differences between sampling time in these intestinal segments ($p \geq 0.017$)
[b]Statistically different when compared to preischemic sample ($p = 0.016$)
M = Missing data

The concept of reperfusion injury was introduced following the observation that reperfusion of ischemic intestine produced much more damage than that caused by a period of ischemia alone (14–15). Further studies suggested that although ischemia potentiates tissue damage, it may actually be the re-entry of oxygenated blood into the ischemic tissue that results in the most damage (21–24). Reactive oxygen and hydroxyl radicals have been implicated as mediators of tissue damage during reperfusion in several organ models, including the myocardium, cerebrum, kidney, intestinal tract and lungs (25–31). In rats it has been shown that, at least in the small intestine, XO mediation of the production of superoxide radicals is responsible for significant endothelial and epithelial damage (19).

It has been hypothesized that, initially, complete ischemia starves the cell of oxygen and glucose, thereby removing the main energy source. This leads to adenosine monophosphate (AMP) production. The AMP ordinarily is catabolized to adenosine, inosine, and finally to hypoxanthine by xanthine dehydrogenase (15). Under ischemic conditions, XDH is converted to XO — leading to the production of superoxide radicals (15).

To our knowledge, the level of total xanthine dehydrogenase plus xanthine oxidase activity has not been determined previously in the equine small intestine. This study revealed that in nonischemic equine small intestine, the location of the intestinal segment influences the level of total enzymatic activity of the XDH and XO system. This is consistent with determinations made in laboratory animals (19). Also consistent with measurements in laboratory animals, we found a nearly threefold increase in the level of total enzyme activity (XDH + XO) from ileum to duodenum (19). Comparable to laboratory animals (19) in which the level of % XO activity in nonischemic small intestine is 19% of the total activity, in horses we found the median level of % XO activity ranging from 24% to 28%. As in laboratory animals (19) there was no significant difference in % XO activity between various segments of nonischemic small intestines. Clearly, the combined level of XDH plus XO enzyme activity in nonischemic equine small intestine is in the range of other species (Table IV) in which reperfusion injury has been documented (19).

TABLE IV. Level of total XDH + XO activity in nonischemic small intestine in mammals

Species	Level of total XDH + XO activity in nmol $min^{-1}g^{-1}$
Humans[a]	29–56
Dogs[a]	120–187
Cats[a]	79
Rats (19)	405–470
Mice[a]	90–210
Cows[a]	120
Horses[b]	48–134

[a]Parks, DA: personal communication, February 1990
[b]This study

The conversion of XDH to XO is the rate-limiting step in the production of superoxide radicals (19). It was reported originally that this irreversible conversion occurred in 1 min (32). More recently, it was reported that conversion of XDH to XO occurs at a rate of 13% per hour (19). The results of our study are in partial agreement with this latter study as the conversion rate was approximately 10% for the first hour ($p = 0.016$). Two hours of ischemia raised the median level of % XO from 27% to 41% but this did not reach statistical significance ($p = 0.08$). Perhaps the two missing samples in the distal small intestine influenced this analysis. There was a tendency ($p = 0.06$) toward an effect of the anesthetic and surgical procedures as the median % XO increased in the control segments from 27% to 32%.

In summary, in the nonischemic equine small intestine, there is an important level of XDH plus XO enzyme activity (XO contributing 24 to 28% of the enzyme activity). After 1 h of ischemia, conversion from XDH to XO occurred. These findings may partially explain the reperfusion injury previously described in the equine small intestine (16).

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