Chemiluminescence of ischaemic and reperfused intestine *in vivo*

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SUMMARY Low level chemiluminescence of exposed rat intestine was measured during occlusive ischaemia and reperfusion. Spontaneous emission of *in vivo* rat intestine (10 ± 1 cps/cm²) decreased almost to zero in animals subjected to ischaemia and when the period of ischaemia lasted only two minutes, chemiluminescence increased beyond control levels (39%, three minutes after reperfusion) at intestine deligation. This overshoot did not occur when rats were pretreated with allopurinol (40+100 mg/kg bw). The ratio of xanthine dehydrogenase to xanthine oxidase activities was 3.46 in preischaemic intestine samples. The same ratio was changed to 0.35 in samples subjected to two minutes of ischaemia. As chemiluminescence appears to reflect the steady state level of singlet oxygen, which in turn derives from the steady state level of peroxy radicals, these results agree with the view that oxygen radicals derived from the xanthine oxidase reaction are involved in the cellular damage produced after ischaemia and reoxygenation in the intestine.

Intact organ chemiluminescence seems to reflect the steady state level of peroxy radicals under physiological conditions.^{1,4} These peroxy radicals are intermediates in the free radical chain reaction of lipoperoxidation, usually initiated by oxygen radicals.^{1,3}

McCord *et al* have recently described xanthine dehydrogenase (XD) that rapidly converts into xanthine oxidase (XO) in non-perfused ileum.^{5,6} It has also been postulated that xanthine may accumulate during ischaemia, as a result of ATP degradation. This, upon reoxygenation of the tissue, will support superoxide anion and hydrogen peroxide production which in turn may initiate a chain of free radical reactions leading to potential cell damage.⁷ The XO inhibitor allopurinol prevents damage during the ischaemia reperfusion stress,⁸ as should be expected if XO is involved in this process.

This study describes the early changes occurring in the *in vivo* intestine chemiluminescence during short term ischaemia and reperfusion.

Methods

EXPERIMENTAL DESIGN

Male Long Evans rats (220-280 g) were anaesthetised with sodium pentobarbital (50 mg/kg bw, ip). A small medial incision was made in the abdominal cavity and a large section of intestine (jejunum) extracted and kept exposed. The rats were placed in a temperature regulated (30°C) light tight box in a photon counter.¹ Short term occlusive ischaemia was induced by ligation of the proximal and distal segments of a mass of small intestine including the mesenteric vessels, by way of an intestinal strangulation, as reported by Frantzides *et al*.⁹ This ligation was carried out at the abdominal incision level, thus affecting the whole section of exposed small intestine. The ischaemic period was maintained for two minutes as it was intended to study early effects. Deligation was performed without affecting the exposure of the measured area.

Upon deligation, animals (about 20% of the total) not showing a complete change of intestinal colour from violet blue to bright red or pink or with macroscopic findings of blood at the site of ligation (bleeding) were excluded from the statistical results,

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as both were considered signs of disturbed reperfusion. During the experimental period no animals died.

A group of rats was pretreated with allopurinol (4-hydroxypyrazol (3,4-d) pyrimidine, Sigma Chemical Co) 1% (w/v) suspension at pH 10.0 and immediately administered ip in two consecutive doses, 40 and 100 mg/kg bw, were given 24 and three hours before ligation.

CHEMILUMINESCENCE MEASUREMENTS

Chemiluminescence was determined using a Johnson Foundation photon counter (Johnson Research Foundation, University of Pennsylvania, Philadelphia, PA, 19714, USA) with an EMI 9658 red sensitive phototube as described by Boveris *et al.*¹² In brief, the intestine surface to be measured for chemiluminescence (12–15 cm²) was placed over a non-emissive surface of aluminium foil, through a small centred window. Aluminium foil was used to cover the rat body and the only area exposed for photoemission was the portion of intestine over the foil. The intestines were placed 10 mm below the optical coupler of the photon counter.¹ The EMI 9658-B phototube is sensitive to light emission in the 300–900 nm range. Chemiluminescence was the difference between dark current (shutter closed) and emission (shutter open) and expressed as counts per second

per cm² (cps/cm²). Spontaneous chemiluminescence of exposed intestine was considered as the photoemission measured before the ligation (control value). Due to the fact that the light-tight box is opened during the ligation-deligation procedure, constant measurements are started after two minutes postdeligation to allow for stabilisation of the tracings.

XANTHINE DEHYDROGENASE AND XANTHINE OXIDASE MEASUREMENTS

Pieces of non-ligated and ligated intestine (jejunum) from other similar groups of rats were placed with a cold buffer containing 140 mM KCl, 20 mM Tris-HCl, pH 7.6, 1.0 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co, a potent serine protease inhibitor) and 10 mM dithioerythritol (to prevent the conversion of xanthine dehydrogenase into the oxidase form) and then homogenised and centrifuged for 10 minutes at 10 000 rpm. The supernatant (200 µl) was placed with 2.9 ml 0.1 M Tris-HCl. Measurements of xanthine oxidase activity were done with a Perkin Elmer 356 spectrophotometer at 292 nm (uric acid appearance) with 0.1 mM xanthine. Xanthine dehydrogenase was measured in the same reaction medium plus 0.1 mM NADH and at 340 nm, as described by Batelli *et al.*¹⁰ Activities are expressed as mU/100 mg of protein and as xanthine

Figure Chemiluminescence (cps) of rat intestine exposed spontaneously (a), under ischaemia (b), under reperfusion (c), and under reperfusion in allopurinol pretreated rats (d).

dehydrogenase/xanthine oxidase ratio. Protein was determined by the method of Lowry *et al*¹¹ with bovine serum albumin as a standard.

STATISTICAL ANALYSIS

Statistical analysis was done through ANOVA one way analysis of variance and *t* test for independent measurements with a *p* value for two-sided test, using a data gathering and analysis system for IBM PC. Values are given as mean (SE).

Results

The spontaneous low level chemiluminescence from exposed intestine (10 ± 1 cps/cm²) remained constant over a period of 20 or more minutes (Figure (a)). Chemiluminescence decreased to 55% and 32% of the original emission three and five minutes after ligation, respectively. Emission decreased to 20% of the original emission in six to eight minutes and to almost zero after 10 to 12 minutes of ischaemia (Figure (b)).

Reperfusion of rat intestine after two minutes of ischaemia produced an overshoot of chemiluminescence (Figure (c)) beyond its control values. This increase was 39% and 25% above control values after three and five minutes of reperfusion, respectively. Intestine from rats pretreated with allopurinol did not show the overshoot and emission levels did not differ from control values (Figure (d)). The average changes in spontaneous chemiluminescence from controls and allopurinol treated rats are shown in Table 1.

Xanthine dehydrogenase and xanthine oxidase activities in normal and ligated intestine are shown in Table 2. An XD/XO mean ratio of 3.46 (0.38) calculated for non-ligated intestine was inverted to a 0.35 (0.10) mean ratio in samples subjected to two minutes of ligation.

Table 1 Effect of ischaemia and reperfusion on spontaneous chemiluminescence from rat intestine *in vivo*

Treatment	Time (min)	n	Treat/control* (mean (SEM))
Ischaemia	3	8	0.55 (0.07)
Ischaemia	5	8	0.32 (0.07)
Reperfusion	3	8	1.40 (0.08)†
Reperfusion	5	8	1.25 (0.07)‡
Reperfusion after allopurinol	3	8	0.89 (0.07)†
Reperfusion after allopurinol	5	8	0.92 (0.04)‡

*Ratio of treatment values versus control values of intestine chemiluminescence, n=eight rats for each condition. Statistic: ANOVA, F: 33.9 *p*<0.001 between groups at three minutes and F: 59.3 *p*<0.001 between groups at five minutes. †T: 4.77 *p*<0.001; ‡T: 4.06 *p*<0.002.

Table 2 Xanthine dehydrogenase (XD) and xanthine oxidase (XO), activities in intestine of rats under control and ischaemic (two minutes of ligation) conditions

Intestine	XD (mU/100 mg)	XO (mU/100 mg)	Mean XD/mean XO
Control (n: 5)	45.1 (5.6)	13.0 (5.1)	3.46
Ischaemia (n: 5)	5.1 (1.4)	14.1 (4.1)	0.35

(1) Comparison of XD/XO ratios between groups. T: 7.97 *p*<0.001. Values are given as mean (SEM). n=five in each group.

Discussion

Experiments carried out by Roy and McCord⁶ have shown that the enzyme XD undergoes an irreversible transformation into XO during ischaemia. The difference between the two enzymatic forms is that the latter generates superoxide anion during the oxidation of xanthine to uric acid, starting a chain of free radical reactions that could be responsible for the cellular damage occurring during reperfusion. This has also been described experimentally in other ischaemia-reperfusion conditions such as myocardial ischaemic injury,^{12,13} shock,¹⁴ organ preservation,¹⁵ island skin flaps,¹⁵ warm renal ischaemia,^{14,16} etc. Supporting this hypothesis are the results obtained by Granger *et al*,⁸ showing no damage in reperfused intestine of rats pretreated with the XO inhibitor allopurinol before the ischaemic injury. The free radical production has been described as an early event in myocardial tissue of rabbits, where an oxygen centred free radical signal is obtained at 10 seconds after reflow with electron paramagnetic resonance technique.¹⁷ Also an increased production of plasma hydrogen peroxide is observed in patients immediately after bypass surgery.¹⁸ In this work an early radical production is also observed in reperfused intestine *in vivo*.

The experiments described here were designed to determine the early role of free radicals during intestinal ischaemia and reperfusion using a non-invasive method with respect to the organ, such as the determination of spontaneous chemiluminescence, which appears to measure the steady state level of singlet oxygen³ and that correlates with oxidative stress and lipid peroxidation.¹⁴ There was a quantitative increase in spontaneous liver chemiluminescence on ethanol treatment quantitatively parallel to malonaldehyde and conjugated diene formation.⁴

Spontaneous chemiluminescence decreased after ligation as the tissue oxygen supply was stopped (Figure (b)). Despite mass ligation of intestine and

mesenteric vessels, this methodology may cause only a partial ischaemia, depending on some intramural circulation. Ischaemia was enough, however, to induce changes in the measured chemiluminescence.

This decrease depended on ischaemia as no major change was found in chemiluminescence for more than 20 minutes of organ exposure when normal circulation continued.

During reperfusion an overshoot of chemiluminescence (instead of a progressive return to control levels) was observed (Figure (c)), suggesting an increased generation of free radicals within the affected tissue. This may reflect saturation of the intracellular antioxidant defences as a consequence of the oxygen radicals generated after the accumulation in hypoxanthine and xanthine and the activation of xanthine oxidase.

The intestine contains a large amount of XD and in experimental ischaemia the rate of its conversion into XO is faster than in any other organ. Roy *et al* have estimated a four seconds mean conversion speed.⁶ The early *in vivo* phenomena described in this paper are consistent with such time course. Further work should show the consequent pathological implication of these phenomena.

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