J. Pharm. Pharmacol. 1994, 46: 519–520
Received July 22, 1993
Accepted December 31, 1993

Evaluation of free radical production in an ischaemia-reperfusion model in the rabbit using a tourniquet

J. CHOPINEAU, M. F. SOMMIER, V. SAUTOU, *Laboratoire de Pharmacie Clinique et Biotechnique, Faculté de Pharmacie, Place Henri Dunant-BP 38, 63003 Clermont-Ferrand Cedex 1, France*

Abstract—Use of a tourniquet on a limb to stem blood flow is frequent in surgery. However, this results in an ischaemia-reperfusion effect that causes damage through the production of free radicals. The aim of this work was to evaluate this production of free radicals in an ischaemia-reperfusion model in the rabbit after placement of a tourniquet. Plasma lipoperoxides were measured by high-performance liquid chromatography using the malondialdehyde assay method. We found significant ($P < 0.01$) production of free radicals 1 min after release of the tourniquet compared with controls; no variation in levels of free radicals was observed during the ischaemia phase.

Free radicals are formed during the normal life of a cell. They result from the single-electron reduction of molecular oxygen. These chemical species have unpaired electrons and so are highly reactive and very short-lived (10^{-11} s) (Halliwell et al 1992). Oxygenated free radicals can react with polyunsaturated fatty acids in membrane phospholipids to form lipid peroxides by enzymatic or non-enzymatic routes (Emerit et al 1991). Under normal conditions, the production of these peroxides is controlled by protective physiological systems such as cytosolic enzymes, antioxidants (Dargel 1991; Pré 1991). These systems of protection may often be insufficient, in particular during ischaemia-reperfusion. In orthopaedic surgery, the tourniquet is vital for controlling intra-operative bleeding. Placement and release of the tourniquet reproduces ischaemia-reperfusion. Using an experimental animal model, we set out to reproduce this situation observed in orthopaedic surgery, particularly of the knee. Our purpose was to assess the production of free radicals during both ischaemia and subsequent reperfusion.

Materials and methods

Free radical assay. To measure the quantity of free radicals produced we used the malondialdehyde assay technique. The lipoperoxides formed by peroxidation of the free radicals are converted into malondialdehyde, which reacts with thiobarbituric acid to form a coloured complex. Malondialdehyde was assayed by HPLC with fluorimetric detection.

Animals. Twenty male New Zealand rabbits, 2500 g, were divided randomly into two groups. One group was subjected to experimental ischaemia by means of a tourniquet (tourniquet group); the other was the control group.

Animal experiments. All rabbits were placed in individual cages. A catheter (V, 22 gauge, Viggo-Spectramed, Helsinborg, Sweden) was placed in the marginal vein of the right ear of the rabbit after vasodilatation with chloroform (Coopérative Pharmaceutique Française, Melun, France). A first blood sample was collected into a 3-mL tube using a 2-mL glass syringe (Luxblind, Ico, Italy) rinsed beforehand in 0·5 M EDTA solution. A rubber elastic tourniquet was placed on the upper right forelimb of the rabbits in the tourniquet group and was kept on for 30 min. A second blood sample was taken 5 min before release of the tourniquet under the same conditions as the first sample. Third and fourth samples were taken, respectively, 1 and 10 min after release of the tourniquet. After each of the four samples, the catheter was rinsed with 0·2 mL sodium heparin solution (333 int. units, 5 mL, Roche, France). Before blood collection, the first 0·5 mL was discarded to remove all traces of heparin.

Correspondence: J. Chopineau, Laboratoire de Pharmacie Clinique et Biotechnique, Faculté de Pharmacie, Place Henri Dunant-BP 38, 63003 Clermont-Ferrand, Cedex 1, France.

Sample preparation. Fifty microlitres of plasma was hydrolysed with 750 μL 0·44 mM phosphoric acid (Merck); 250 μL 42 mM thiobarbituric acid and 450 mL distilled water were added and the mixture was heated in a boiling-water bath for 1 h. The hydrolysis product obtained, malondialdehyde, reacts with thiobarbituric acid to form a coloured complex. The reaction was quenched in ice and the samples were neutralized with equal parts of sodium hydroxide-methanol (4·5–46·5 v/v) and spun for 5 min at 4500 rev min^{-1}.

Chromatographic analysis. The HPLC system was comprised of a series of Merck-Hitachi components: L6200 Intelligent pump, rheodyne injector valve, F 1050 fluorimetric detector and D 2500 integrator. The analysis column was an endcapped Merck Lichrospher 100 RP18. The mobile phase was a 40/60 v/v mixture of methanol and 50 mM pH 6·8 potassium phosphate buffer. The flow rate was 1·3 mL min^{-1}; under these conditions the malondialdehyde-thiobarbituric acid complex had a retention time of 1·8 min. The excitation wavelength was set at 520 nm and the emission wavelength at 550 nm. The injection volume was 20 μL.

Calibration was performed using dilute solutions of 1,1,3,3-tetraethoxypropane (TEP, Sigma Chemical Company, St Louis, USA) in the concentration range 0·61–4·86 μM, which on hydrolysis releases stoichiometric quantities of malondialdehyde.

Statistical analysis. The statistical analysis consisted of the comparison of tourniquet groups between the different samples.

Results and discussion

Malondialdehyde assay. The retention time of malondialdehyde was 1·85 ± 0·04 min (CV = 2·2%, n = 10). The assay afforded

Table 1. Malondialdehyde in rabbit plasma after application of a tourniquet for 30 min.

Sampling time	Control	Experimental
Before application of tourniquet	2·13 ± 0·46	2·37 ± 0·43
After 25 min	1·96 ± 0·33	2·01 ± 0·42
1 min after removal	2·00 ± 0·40	2·49 ± 0·70*
10 min after removal	2·19 ± 0·47	2·23 ± 0·68**

* $P < 0.01$ compared with 25-min sample, ** $P < 0.04$ compared with 1-min sample. There was no significant difference between plasma concentrations before tourniquet application and 10 min after removal, or between plasma concentrations before tourniquet application and before its removal.

adequate linearity for concentrations in the range 0·61–4·86 μM with a correlation coefficient of 0·999.

The precision of the method was evaluated by means of intra-day (8·9% at 0·61 μM, 1·5% at 4·86 μM) and inter-day (6·1% at 0·61 μM, 1·6% at 4·86 μM) variabilities. The detection threshold was 0·05 μM.

Animal experiments. The results are summarized in Table 1, and indicate there is an increase in free radicals only during the reperfusion phase immediately following removal of the tourniquet.

It has been shown that cell lesions are more serious when the ischaemic tissue undergoes rapid re-oxygenation (Kloner 1993). We found that the level of free radicals rose very soon after the start of reperfusion; 1 min after release of the tourniquet, formation of free radicals was significant.

The conflicting effects of reperfusion subsequent to acute ischaemia is a subject of much debate in clinical practice (Menger et al 1991). An ischaemic area has to be reperfused since non-perfusion leads to cell hypoxia, which can cause necrosis. However, reperfusion is accompanied by the production of oxygenated free radicals, which place the cells under oxidative stress with consequences outside the site of the stress. The physiological free-radical-scavenging system is also evoked throughout the organism (Emerit et al 1991). It would be of interest to investigate whether radical scavengers (superoxide dismutase, ascorbic acid, α-tocopherol, xanthine oxidase inhibitor) could forestall effects due to reperfusion, and so be useful in clinical applications such as orthopaedic surgery.

The authors thank Françoise Picq and Hélène Confolent for the malondialdehyde assay.

References

Dargel, R. (1991) Lipid peroxidation—a common pathogenetic mechanism? Exp. Toxicol. Pathol. 44: 169–181

Emerit, J., Klein, J. M., Coutellier, A., Coney, F. (1991) Radicaux libres et peroxydation lipidique en biologie cellulaire: perspectives physiopathologiques. Path. Biol. 39: 316–327

Halliwell, B., Gutteridge, J. M. C., Cross, C. E. (1992) Free radicals, antioxidants, and human disease: where are we now? J. Lab. Clin. Med. 119: 598–620

Kloner, R. A. (1993) Does reperfusion injury exist in humans? J. Am. Coll. Cardiol. 21: 537–545

Menger, M. D., Lehr, H.-A., Messmer, K. (1991) Role of oxygen radicals in the microcirculatory manifestations of postischemic injury. Klin. Wochenschr. 69: 1050–1055

Pré, J. (1991) La lipoperoxidation. Path. Biol. 39: 716–736

J. Pharm. Pharmacol. 1994, 46: 520–521
Received October 27, 1993
Accepted January 27, 1994

Prolonged anti-emetic activity and 5-HT_3-receptor antagonism by BRL 46470 in conscious ferrets

J. BERMUDEZ, G. J. SANGER, *SmithKline Beecham Pharmaceuticals, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD, UK*

Abstract—The anti-emetic activity of oral and intravenously-administered BRL 46470 (endo-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3yl)-2,3-dihydro-3,3-dimethyl-indole-1-carboxamide HCl) has been assessed in conscious ferrets. BRL 46470 (0·05–0·5 mg kg^{-1}, p.o.) dose-dependently prevented emesis evoked over a 2 h period by total body X-irradiation. This anti-emetic activity occurred with oral or intravenously-administered BRL 46470 even when dosed 3–4 h before radiation. In conjunction with data obtained in other species, we conclude that BRL 46470 has a potent and long-lasting ability to antagonize actions that are mediated by the 5-HT_3 receptor in-vivo.

BRL 46470 is a potent, highly selective 5-HT_3-receptor antagonist with a long duration of action in anaesthetized rats and a demonstrable anxiolytic-like activity in conscious rats (Blackburn et al 1993). We now report on the ability of BRL 46470 to prevent emesis evoked by total body X-irradiation in ferrets, a pathological event which in this animal is wholly sensitive to 5-HT_3-receptor antagonism (Sanger 1993). Our results support the view that BRL 46470 has a prolonged and highly potent ability to antagonize at the 5-HT_3 receptor in-vivo.

Materials and methods

The techniques have been previously described (Bermudez et al 1988). Male ferrets, either polecat or albino, 1–2 kg, were used.

Correspondence: G. J. Sanger, SmithKline Beecham Pharmaceuticals, Coldharbour Road, The Pinnacles, Harlow, Essex CM19 5AD, UK.

Emesis was induced by total body X-irradiation (10·4 min, at approx. 300 rads min^{-1}). Animals were then observed for the time of onset of emesis (the latency period) and for the number of emetic episodes within the monitoring period (2 h).

Statistics. The results are given as means ± s.e.m. and were analysed statistically using the Student's *t*-test for unpaired data.

Chemicals. BRL 46470 (endo-*N*-(8-methyl-8-azabicyclo[3.2.1]oct-3yl)-2,3-dihydro-3,3-dimethyl-indole-1-carboxamide HCl) was synthesized in-house and was dissolved in 0·9% NaCl.

Results

Oral administration of BRL 46470 dose-dependently prevented radiation-evoked emesis (Table 1). Oral pre-dosing with BRL 46470 1 and 3 h before radiation, prevented or greatly reduced emesis throughout the subsequent 2 h observation period; pre-dosing by 4 h only partially reduced emesis (Table 2). BRL 46470 (0·5 mg kg^{-1}) injected intravenously 3 h before radiation also prevented emesis throughout the subsequent 2-h observation period. However, when this dose of BRL 46470 was injected 4 h before radiation, emesis was greatly reduced but not totally prevented.