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Hyperoxia and salicylate metabolism in rats

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Abstract—Following the administration of aspirin to rats by gavage, both 2,3- and 2,5-dihydroxybenzoate appeared in the plasma. The concentration of the 2,3-dihydroxybenzoate was significantly higher in animals exposed to 100% O_2 for 60 h than in air breathing controls. These data are evidence that increased salicylate hydroxylation is associated with oxidative stress.

The anti-inflammatory activity of aspirin (acetylsalicylic acid) and other non-steroidal anti-inflammatory drugs has been attributed to their ability to inhibit cyclo-oxygenase activity and hence impair hydroxyl radical (OH·) release from polymorphonuclear leucocytes (Sagone et al 1980). However, aspirin is rapidly hydrolysed to salicylate in-vivo (Levy 1979) and while this metabolite has anti-inflammatory properties it does not inhibit cyclo-oxygenase (Vargaftig 1978). It is possible that a major contribution to the anti-inflammatory capacity of salicylates is their ability to scavenge free radicals. Such compounds will protect bacteriophage viability in exposure to OH· generated in γ-radiolysis (Hiller & Wilson 1983), and activated human granulocytes have been shown to oxidize salicylate in-vitro, apparently through the release of OH· (Sagone & Husney 1987).

If salicylate is able to scavenge OH· in-vivo then it should be possible to detect products of this reaction. It is known that exposure of salicylate to hydroxyl free radicals in-vitro will generate approximately equal amounts of 2,3- and 2,5-dihydroxybenzoates together with some catechol (Grootveld & Halliwell 1986). 2,5-Dihydroxybenzoate (gentisate) is known to be a significant metabolite of salicylate in normal subjects and plasma levels were not significantly raised in rheumatoid arthritis patients (Cleland et al 1985). 2,3-Dihydroxybenzoate is present in only trace amounts in normal subjects taking aspirin and may be a product of background radiation. Three patients with rheumatoid arthritis were found to have increased plasma concentrations of this metabolite (Grootveld & Halliwell 1986). To our knowledge there have been no further studies of 2,3-dihydroxybenzoate in oxidative stress in-vivo.

The aim of the present study was to determine whether increased hydroxylation of salicylate could be detected in an animal model of free radical mediated injury. There is good evidence that the lung damage which occurs in hyperoxia is mediated by a free radical mechanism. For example, superoxide dismutase is induced in the lungs of rats exposed to 90% O_2 (Kimball et al 1976) and such exposure confers increased tolerance to the lethal effects of 100% O_2 (Freeman et al 1982). Plasma concentrations of dihydroxybenzoates were therefore measured following the administration of aspirin to rats which were exposed to 100% O_2.

Materials and methods

Soluble aspirin tablets (Solprin) were supplied by Reckitt and Colman. Standard solutions of 2,3-, 2,5- and 3,4-dihydroxybenzoate (Sigma) in 50 mmol L^{-1} HCl were diluted in water as required. Rats (Sprague-Dawley, female, approx. 200 g) were exposed to 100% oxygen or air (for 24, 48 or 60 h) in a large Perspex container into which a conventional cage containing 6 rats could be placed. Oxygen was delivered at a flow rate of 15 L min^{-1}. Slight positive pressure was maintained to prevent ingress of air. Soluble aspirin was administered by gavage (50 mg in 1 mL of water). In pilot experiments blood was taken by cardiac puncture under terminal anaesthesia, either 1, 2 or 3 h after gavage. In oxygen exposure experiments animals were given aspirin by gavage 2 h before the end of the exposure period. Blood was again taken by cardiac puncture under terminal anaesthesia (2 h after gavage).

Samples were prepared and analysed by HPLC, essentially as described by Grootveld & Halliwell (1986). Internal standard (100 μL of 10 μmol L^{-1} 3,4-dihydroxybenzoate) and HCl (50 μL of 1 mol L^{-1}) were added to 1 mL of plasma. The plasma was extracted twice with 8 mL of ethylacetate (vortex mixing for 60 s). The organic phases were pooled and evaporated. The solid was dissolved in water (200 μL) plus HCl (50 μL of 1 mol L^{-1} with vortex mixing (30 s) with the aid of a glass bead. Samples were then passed through a 0·45 μm disposable filter (Gelman Sciences ACRO LC13) and stored at −20°C.

A spherisorb 5 ODS reverse-phase HPLC column (250 × 4·6 mm) was eluted with 30 mmol L^{-1} sodium citrate/acetate buffer (pH 4·65). Metabolites were detected electrochemically with an EDT LCA 15 detector equipped with a glassy carbon working electrode and a Ag/AgCl reference electrode. Dihydroxybenzoates were identified by comparison with the elution times of standards. Quantification was by direct comparison of peak heights with that of the internal standard. Plasma salicylate was determined by the method of Trinder (1954). Statistical analysis was by one way analysis of variance, and *t*-tests where appropriate.

Results

Aspirin (50 mg) given to rats by gavage achieved plasma salicylate concentrations of 1·96, 1·80 and 1·82 mmol L^{-1} at 1, 2 and 3 h, respectively (data are the means of 3 separate experiments). In all subsequent experiments aspirin was given 2 h before the animals were killed. Plasma salicylate concentrations in animals exposed to 100% O_2 for 24, 48 or 60 h were not significantly different from those of control animals (Table 1).

Plasma 2,3-dihydroxybenzoate was significantly higher in animals exposed to 100% O_2 for 60 h compared to controls whether expressed as absolute concentration or relative to salicylate (Table 1). No significant change in this metabolite was found after 24 or 48 h exposure to 100% O_2. No significant changes in 2,5-dihydroxybenzoate were found at any exposure time.

Discussion

It is clear that salicylates are good free radical scavengers, with rate constants for reaction with OH· of about 5×10^9 M^{-1} s^{-1} (Hiller & Wilson 1983). Since body fluids comprise a range of molecules including endogenous antioxidants and proteins, which also react rapidly with OH·, it is difficult to predict whether salicylate will scavenge significant amounts of free radical in-vivo. The results presented here show that there is

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Table 1. Metabolites in plasma of hyperoxic rats given aspirin by gavage.

	Time (h)			
Metabolite	60 (Air)	24 (100% O_2)	48 (100% O_2)	60 (100% O_2)
Salicylate (mmol L^{-1})	1·76 (±0·05)	1·81 (±0·11)	2·09 (±0·17)	2·01 (±0·07)
2,3-Dihydroxybenzoate (mmol L^{-1})	0·34 (±0·02)	0·31 (±0·03)	0·45 (±0·07)	[a]0·60 (±0·09)
2,3-Dihydroxybenzoate (mmol mol^{-1} salicylate)	0·19 (±0·01)	0·17 (±0·03)	0·21 (±0·02)	[b]0·30 (±0·04)
2,5-Dihydroxybenzoate (mmol L^{-1})	3·81 (±0·63)	3·77 (±0·46)	4·65 (±0·72)	4·24 (±0·78)
2,5-Dihydroxybenzoate (mmol mol^{-1} salicylate)	2·17 (±0·36)	2·08 (±0·33)	2·42 (±0·42)	2·15 (±0·40)

[a] and [b] significantly higher than air controls ($P < 0·01$ and $P < 0·02$, respectively).
Data are mean ± s.e. (n = 12).

increased hydroxylation of salicylate in rats which had been exposed to 100% oxygen for 60 h, but no significant increase after 24 or 48 h. It has previously been shown that there is a significant increase in the number of neutrophils in the lung capillaries of rats between 40 and 60 h of exposure to 100% O_2 (Barry & Crapo 1985). It is therefore probable that free radicals are generated at this time although this is not confirmed by any change in antioxidant concentrations in adult rats exposed to 100% O_2 (Yam et al 1978; Turrens et al 1984).

Other than the study on samples from three rheumatoid patients (Grootveld & Halliwell 1986), this paper presents the first data on the effect of oxidative stress on salicylate hydroxylation to 2,3-dihydroxybenzoate in-vivo. This link does not establish a mechanism for the increased production of this metabolite, however the findings are consistent with the hypothesis that salicylate can act as a hydroxyl radical scavenger in-vivo. Another possible explanation is that some other metabolic route exists for the formation of 2,3-dihydroxybenzoate, and further studies with other models of oxidative injury are required.

References

Barry, B. E., Crapo, J. D. (1985) Patterns of accumulation of platelets and neutrophils in rat lungs during exposure to 100% and 85% oxygen. Am. Rev. Respir. Dis. 132: 548–555

Cleland, L. G., Lowthian, P. J., Imhoff, D., Bochner, F., Betts, W. H., O'Callaghan, J. (1985) Plasma and sinovial fluid gentisate in patients receiving salicylate therapy. J. Rheumatol. 12: 136–139

Freeman, B. D., Topolsky, M. K., Crapo, J. D. (1982) Hyperoxia increases oxygen radical production in rat lung homogenates. Arch. Biochem. Biophys. 216: 477–487

Grootveld, M., Halliwell, B. (1986) Aromatic hydroxylation as a potential measure of hydroxyl-radical formation in vivo. Biochem. J. 237: 499–504

Hiller, K. O., Wilson, R. L. (1983) Hydroxyl free radicals and anti-inflammatory drugs: biological inactivation studies and reaction rate constants. Biochem. Pharmacol. 32: 2109–2111

Kimball, R. E., Krishna, R., Peirce, T., Schwartz, L. W., Mustafa, M. G. (1976) Oxygen toxicity: augmentation of antioxidant defense mechanism in rat lung. Am. J. Physiol. 230: 1425–1431

Levy, G. (1979) Pharmacokinetics of salicylate in man. Drug Metab. Rev. 9: 3–19

Sagone Jr., A. L., Husney, R. M. (1987) Oxidation of salicylates by stimulated granulocytes: evidence that these drugs act as free radical scavengers in biological systems. J. Immunol. 138: 2177–2183

Sagone Jr., A. L., Wells, R. M., DeMocko, C. P. (1980) Evidence that OH· production by human PMNs is related to prostaglandin metabolism. Inflammation 4: 65–71

Trinder, P. (1954) Rapid determination of salicylate in biological fluids. Biochem. J. 57: 301–303

Turrens, J. F., Crapo, J. D., Freeman, B. A. (1984) Protection against oxygen toxicity by intravenous injection of liposome entrapped catalase and superoxide dismutase. J. Clin. Invest. 73: 87–95

Vargaftig, B. B. (1978) Salicylic acid fails to inhibit generation of thromboxane A_2 activity in platelets after in vivo administration to the rat. J. Pharm. Pharmacol. 30: 101–104

Yam, J., Frank, L., Roberts, R. J. (1978) Oxygen toxicity: comparison of lung biochemical responses in neonatal and adult rats. Pediat. Res. 12: 115–119