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Arteriolar oxygenation in tumour and subcutaneous arterioles: effects of inspired air oxygen content

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Summary Carbogen is thought to be more effective than normobaric oxygen in reducing tumour hypoxia because it may reduce hyperoxic vasoconstriction. In this study, tumour and normal arteriolar diameters were measured simultaneously with perivascular pO_2 during air breathing followed by either carbogen or 100% oxygen to determine whether the action of carbogen is the result of alterations in feeding vessel diameter. Fischer-344 rats bearing dorsal flap window chambers, with or without implanted R3230AC tumours, were the experimental subjects. Arteriolar diameters were measured using optical techniques and perivascular pO_2 was measured using recessed-tip electrodes (3-6 μm tip diameter). Baseline arteriolar pO_2 averaged 30-50% of blood gas pO_2 (mean = 97 mmHg). Both hyperoxic gases increased blood gas pO_2 by 4-to 5-fold, but relative improvements in arteriolar pO_2 were ≤ 2.5 for all arterioles studied. This means that these normobaric high O_2 gases are not very efficient in increasing O_2 delivery to tumours. In addition, improvements in tumour arteriolar pO_2 were transient for both hyperoxic gases. Oxygen and carbogen caused no change and mild vasodilatory responses in tumour arterioles, respectively. Normal arterioles on the other hand, tended toward vasoconstriction by carbogen breathing. Peri-arteriolar pO_2 in tumours increased within the first 5 min of breathing either hyperoxic gas, followed by a decline back toward values seen with air breathing. These results suggest that temporal changes in tumour oxygenation after exposure to carbogen or O_2 may not be due to changes in perfusion. Other factors, such as changes in O_2 consumption rate may be involved.

Keywords: carbogen; hyperoxia; perfusion; vasoactive

Recent theoretical studies by our group suggest that knowledge of tumour arteriolar pO_2 is important in understanding O_2 transport in tumours and that increasing arteriolar pO_2 could reduce hypoxia in some circumstances (Secomb *et al.*, 1995). Carbogen breathing has been advocated as a method to ameliorate O_2-induced vasoconstriction and improve tumour oxygenation (Rojas, 1992). We therefore elected to compare tumour and normal arteriolar pO_2 and diameters under conditions of air, normobaric O_2 and carbogen breathing, using R3230AC tumours grown in dorsal flap window chambers of Fischer-344 rats.

Our results suggest three important features of arteriolar oxygenation. First, blood pO_2 drops steeply from the femoral artery to the terminal arteriole under air breathing conditions. Second, the steepness of the gradient increases when high oxygen content gases are used. Third, our data indicate that the vasoactive effects of these gases in this model system do not correspond to the widely held views about how these gases affect arteriolar tone and perfusion. With both hyperoxic gases, the temporal course of change in tumour arteriolar oxygenation is transient and not coordinated with changes in arteriolar diameter. These results suggest that other mechanisms, such as changes in oxygen consumption rate, may be playing a role in reducing tumour oxygenation after the first few minutes of exposure.

Materials and methods

Animal model

Fischer-344 rats (Charles River Laboratories, Raleigh, NC, USA), were surgically implanted with cutaneous window chambers, using methods previously described (Papenfuss *et al.*, 1979). R3230AC mammary tumours were transplanted into the window chambers at the time of surgery. The animals were housed in an environmental chamber, with normal light-dark cycles and access to food and water *ad libitum* for 9-11 days before experimentation. All protocols were approved by the Duke Animal Care and Use Committee.

Anaesthesia and blood gases

Animals were anaesthetised using i.p. sodium pentobarbital (40-50 mg kg^{-1}) for all surgical and experimental procedures. Rectal temperature was maintained at 37°C using thermostatically controlled blankets (Model 50-7503 Homeothermic Blanket, Harvard Bioscience, N. Natick, Mass., USA). In all experimental procedures, blood pressures and heart rates were monitored using computerised acquisition (AT-codas, Dataq Instruments, Akron, OH, USA) of femoral arterial waveforms (Gould P23XL, Gould Instrument Systems, Cleveland, OH, USA). Blood gas measurements were taken using femoral arterial blood, as previously described (Brizel *et al.*, 1995). Sample volumes did not exceed 0.2 ml and the samples were kept on ice before analysis. Since three samples were taken, the maximum volume removed was 0.6 ml, which is less than 10% of total blood volume of a 150 g rat. For the purposes of reporting of data in this paper, we refer to arterial blood gas measurements as 'blood gas,' rather than 'arterial' so as to avoid confusion with the arteriolar measurements that were made with microelectrodes.

Videomicroscopy

Videomicroscopy of the window chamber was used to observe microvascular parameters during experimental procedures (Zeiss Photomicroscope III, Carl Zeiss, New York, NY, USA). Window chamber arterioles were visualised

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using transillumination (40 W tungsten source). Data were acquired using a CCD camera for transillumination (MTI CCD-72, Dage-MTI, Michigan City, MI, USA) and recorded to videotape (SVO-9500MD, Sony Corporation of America, San Jose, CA, USA). A videotimer image was superimposed on all videotapes for record keeping (CTG-55 Video Timer, For.A. Co., Los Angeles, CA, USA).

Perivascular oxygenation

Oxygen concentration of arterioles was measured using recessed-tip microelectrodes, with tip diameters of 3–6 μm (Dewhirst *et al.*, 1992). Briefly, access to vessels was accomplished by removing one window and suffusing the surface of the window tissue with media bubbled with nitrogen to remove dissolved oxygen. Microelectrodes were positioned next to vessel walls using a micromanipulator (Model MO102E, Narishige Inc., Narishige, Japan). Polarographic current was measured (Chemical Microsensor, Model 1201, Diamond General Development Corporation, Ann Arbor, MI, USA), digitised and continuously recorded on computer using the CODAS software described above.

Arteriolar diameter measurements

Creation of the window chamber leaves a fascial plane of subcutaneous tissue between the windows, which serves as a substrate for tumour implantation. The growing tumour recruits arteriolar feeders from the underlying fascial plane. These vessels were visualised by looking at the fascial plane surface of the window chamber. Tumour arterioles were identified by (1) observable divergent flow; (2) straight wall, with birefringence that is associated with the intimal layer; and (3) the vessels had to be directly connected to microvessels that enter the tumour mass in order to be classified as tumour arterioles (Dewhirst *et al.*, 1994). Arteriolar diameters were measured using an image shearing monitor (Model 907, IPM, Inc., La Jolla, CA, USA).

Experimental protocols

Once each animal was positioned on the microscope stage and the suffusion medium was started, arterioles were identified as described above. In the case of tumour arterioles, the branch closest to the tumour was measured. In non-tumour-bearing preparations arterioles were randomly selected for measurement. The animal was fitted with a face mask to which the various gases could be presented to the animal by being blown across the face. The microelectrode was positioned immediately adjacent to the arteriolar wall. Measurements in this position represent the intravascular pO_2 of arterioles to within 1–2 mmHg and measurement of wall pO_2 avoids the risk of haemorrhage (Duling and Berne, 1970).

Baseline measurements of arteriolar pO_2 were made while the animal was breathing air for a period of 5 min. The gas mixture was then changed to either carbogen or 100% oxygen and additional measurements were taken. For all experiments, the duration of hyperoxic gas exposure was 20 min. Animals were returned to air breathing for a period of 5 min after breathing the high oxygen content gases. Blood gases were repeated after 5 min oxygen breathing, after 20 min hyperoxic gas breathing and 5 min after being returned to room air.

Statistical methods

All quoted *P*-values are two-sided. Two groups of experiments were compared non-parametrically, using the Wilcoxon rank sum test, computing the exact significance level. In most instances, the sample sizes were small, so statistical significance could not be reached using the exact distribution of the Wilcoxon test. In these cases *t*-tests were also used. Changes over time were analysed by modelling differences of pO_2 and the logarithm of the diameter divided by the baseline diameter. No adjustments were made for multiple comparisons of groups at different time points. The models for the repeated measurements contained a random subject effect to account for the correlation of multiple observations made over time on the same animal. The regression models included baseline values (diameter or pO_2) and time (min) as covariates, and were fitted using the SAS mixed procedure. Errors reported are standard error of the mean and are indicated in parentheses after mean values.

Results

Blood pressure and blood gases

Baseline parameters for blood pressure were within normal limits for unanaesthetised rats, averaging around 110 mmHg (Table I) (Smith *et al.*, 1985). Blood gases under air breathing conditions averaged 97 mmHg (pO_2), 46 mmHg (pCO_2), and 7.39 (pH). Blood pressure and blood gas pH were not affected by administration of either carbogen or normobaric oxygen. Blood gas pCO_2 rose slightly during hyperoxic gas exposure, but returned to baseline after re-exposure to air. Blood gas pO_2 rose by approximately a factor of 4.5 during breathing of both high oxygen content gases.

Arteriolar diameter

Under air breathing conditions arteriolar diameters averaged 47.0 (5.9) and 43.1 (3.2) μm for normal and tumour arterioles, respectively. Initial diameters did not differ from each other within subgroups either (data not shown). Both hyperoxic gases tended to induce vasomotor activity. Exaggerated arteriolar vasomotion occurred in about 50%

Table I

Experimental group	*Map*[a]	*Blood gas*[a]			*Baseline peri-arteriolar* pO_2			
					Normal		*Tumour*	
		pO_2	*pH*	pCO_2	*N*	pO_2	*N*	pO_2
100% Oxygen								
Air	110 (3)	96 (5)	7.39 (0.01)	47 (1.2)		50 (7)		22 (6)
100% oxygen at 2 min[b]	111 (5)	448 (11)	7.34 (0.01)	53 (1.8)	6	89 (16)	6	48 (16)
Air	107 (5)	119 (5)	7.43 (0.01)	43 (1.2)		48 (10)		31 (15)
Carbogen								
Air	110 (2)	98 (5)	7.38 (0.01)	46 (1.2)		50 (14)		44 (9)
Carbogen at 2 min[b]	110 (3)	479 (10)	7.35 (0.02)	50 (1.8)	5	65 (12)	5	83 (16)
Air	114 (4)	138 (14)	7.39 (0.01)	45 (1.8)		40 (11)		25 (14)
Summary baseline values[c]	109 (3)	97 (4)	7.39 (0.01)	46 (1)	11	51 (6)	11	32 (6)

[a]Averages from tumour- and non-tumour-bearing animals combined. MAP = mean arterial pressure (pO_2 and pCO_2 in mmHg). [b]Temporal data shown in Figures 3–5. [c]Average of first air breathing measurements.

of vessels studied. Frequencies ranged from 0.7–2.5 cycles min^{-1}, and amplitudes ranged between 10 and 60% of baseline diameter.

In both tissues there was no change in arteriolar diameter during exposure to normobaric oxygen (Figure 1).

The effects of carbogen were different from oxygen. In this case, both arterioles showed evidence of vasoconstriction, but tumour arterioles vasodilated slightly after the first 5 min (Figure 2). The only time points for which these changes were significant between tissues were after 15 and 20 min of carbogen breathing ($P=0.05$ and 0.08 respectively), and at no time were the trends different from zero for either tissue. Changes in normal arterioles bordered on significance at 1, 2, 3, and 4 min ($P=0.06$). These results support the conclusion that carbogen may be slightly vasoconstrictive in this normal tissue and slightly vasodilatory in tumour tissue.

Arteriolar pO_2

All arteriolar pO_2 values were substantially less than arterial blood gas pO_2, which averaged 97 (4) mmHg in air breathing conditions (Table I). Under air breathing conditions peri-arteriolar pO_2 averaged 32 and 51 mmHg in tumour and normal arterioles, respectively. The difference between tissues was significant ($P=0.03$).

Normobaric oxygen breathing caused an increase in normal and tumour arteriolar pO_2, but only during the first 5 min of oxygen breathing (Figure 3). There was not a significant difference in effect between tumour and normal arterioles. During oxygen breathing, blood gas pO_2 averaged 447 (11) mmHg.

Carbogen caused a trend toward improvement in arteriolar oxygenation in both tumours and normal tissues

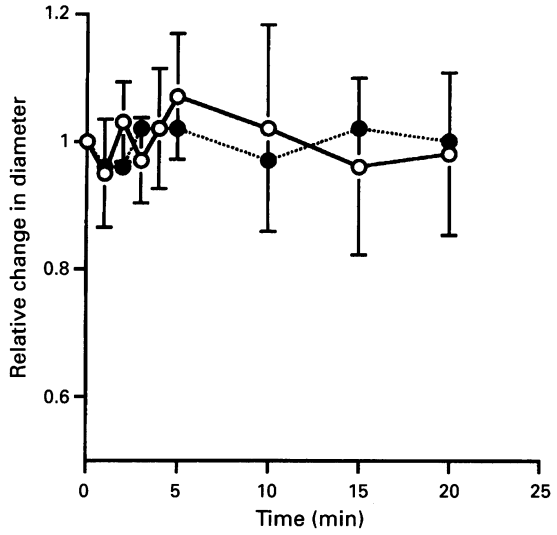

Figure 1 Effects of normobaric oxygen breathing on normal and tumour arteriolar diameter. The observed changes are not significant. Error bars are s.e.m. Normal arterioles (○); tumour arterioles (●).

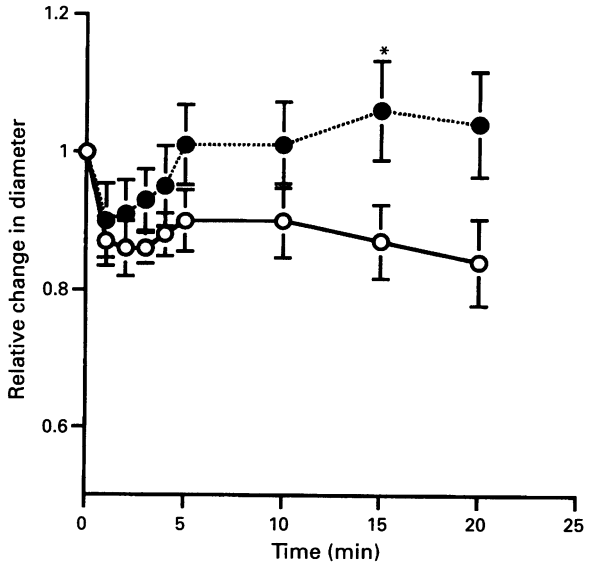

Figure 2 Effects of carbogen breathing on normal and tumour arteriolar diameter. *Difference between tumour and normal significant ($P=0.05$). Changes in diameter of normal arterioles are of borderline significance, relative to baseline ($P=0.06$ at 1–4 min). Error bars are s.e.m. Normal arterioles (○); tumour arterioles (●).

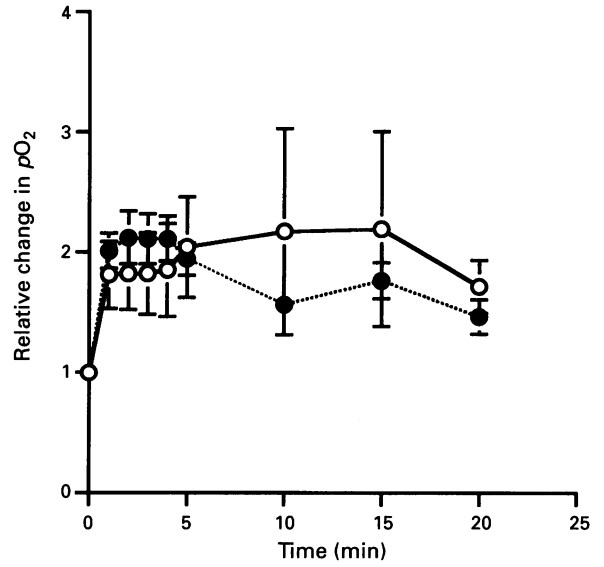

Figure 3 Effects of normobaric oxygen breathing on peri-arteriolar pO_2 of both tumour and normal tissues. Changes from 1–5 min are significant, relative to baseline ($P=0.03$). Error bars are s.e.m. Normal arterioles (○); tumour arterioles (●).

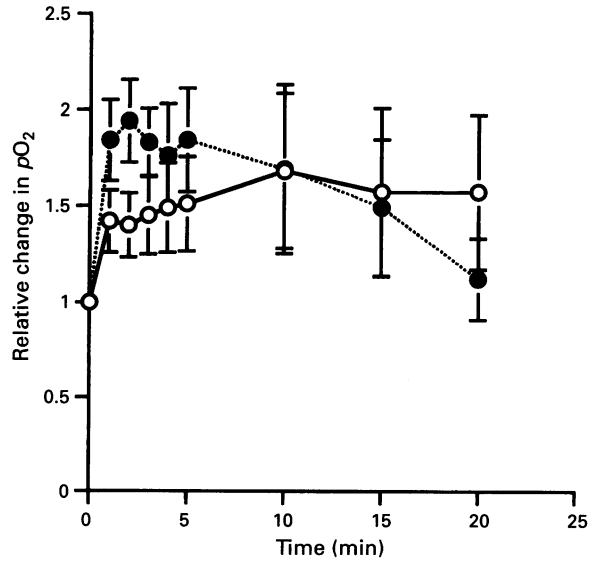

Figure 4 Effects of carbogen breathing on peri-arteriolar pO_2 of both tumour and normal tissues. Changes from 1–5 min are of borderline significance, relative to baseline ($P=0.06$). Error bars are s.e.m. Normal arterioles (○); tumour arterioles (●).

during the first 5 min of gas breathing, but at no time point was the difference statistically significant ($P=0.06$ for all time points) (Figure 4). Arterial blood gas levels averaged 480 (13) mmHg during carbogen breathing.

A comparison between the temporal effects of oxygen and carbogen on tumour arteriolar oxygenation showed that both gases caused an early peak in pO_2 followed by a decline. There was no difference in the effect between the two hyperoxic gases (Figure 5).

Discussion

Peri-arteriolar oxygen gradients in air breathing conditions

A common assumption made in simulations of oxygen transport in tumours has been that arteriolar pO_2 in tumours approximates that of arterial pO_2 (referred to as blood gas pO_2 in this paper), the latter being obtained from large arteries, such as the aorta or femoral artery (Degner and Sutherland, 1988; Groebe and Vaupel, 1988; Secomb *et al.*, 1995). This study demonstrates that this assumption is not true, at least for this tumour model. There is precedent for the existence of peri-arteriolar oxygen gradients in normal tissues as well, so it is likely that peri-arteriolar gradients such as the ones seen here are typical of many tumours. Duling and Berne, 1970 measured arteriolar pO_2 gradients in hamster and rat cremaster muscles. When blood gas pO_2 averaged 79 mmHg, the average pO_2 of 'small arteries' (60–100 μm diameter) was 35 mmHg. The pO_2 of terminal arterioles (10–20 μm diameter) averaged 21 mmHg. Thus, in these tissues, arteriolar pO_2 was between 33 and 50% of blood gas pO_2. The arteriolar pO_2 in our studies ranged between 30 and 50% of blood gas pO_2, but our baseline arteriolar pO_2 values were slightly higher than those reported by Duling and Berne. This is probably due to two factors. First, baseline blood gas pO_2 levels were higher in our study and second, the average arteriolar diameter is larger (42–45 μm) than the terminal arterioles studied in the prior paper.

In a more recent study, haemoglobin saturation measurements were made to assess the degree of deoxygenation that occurs in arteriolar and venular branches of resting muscle tissue (Swain and Pittman, 1989). These analyses demonstrated that the majority of oxygen lost from haemoglobin occurs from the arterial tree, not in capillaries. In particular, they found that haemoglobin saturation of 60 μm arterioles averaged 70% and dropped to 57% in terminal arterioles. In contrast, haemoglobin saturation only fell by an additional 7% when traversing from terminal arterioles to collecting veins. From these data it was estimated that two-thirds of oxygen is lost from haemoglobin in the arterial end of the vascular tree. Our data suggest that similar effects occur in these window chamber preparations in both tumour and non-tumour-containing preparations.

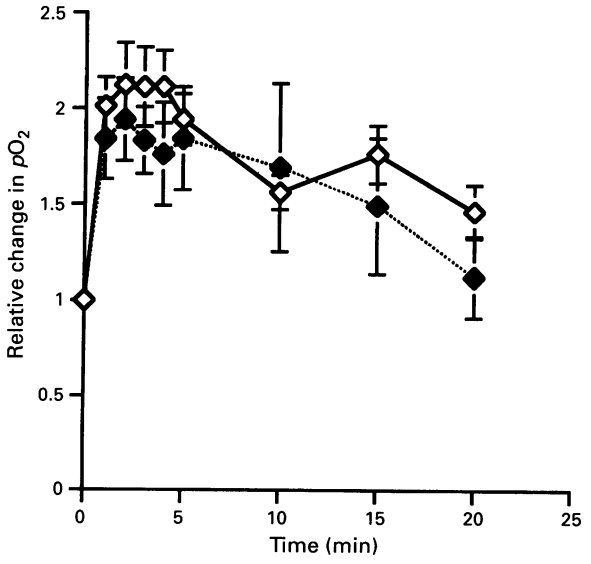

Figure 5 Direct comparison of arteriolar pO_2 in tumour arterioles as a function of oxygen or carbogen exposure. Peri-arteriolar pO_2 increased in both types of vessels, followed by a return toward levels achieved during initial air breathing conditions. Oxygen (◇); carbogen (◆). Error bars are s.e.m.

Effects of carbogen and oxygen on peri-arteriolar oxygen gradient

The overall effect of normobaric high oxygen gases in increasing arteriolar pO_2 was proportionally less than the relative increase in blood gas pO_2. This means that the gain in arteriolar pO_2 by administration of such gases is less than might be expected. The relative increase in blood gas pO_2 was near a factor of 4.5 for both carbogen and oxygen. During oxygen breathing, the pO_2 of normal and tumour arterioles increased 2-fold (Figure 3). With carbogen breathing, normal and tumour arteriolar oxygen concentrations increased by a factor near 1.5 (Figure 4). In previous studies in hamster cremaster muscle, exposure to 95% oxygen and 5% nitrogen increased terminal arteriolar pO_2 from 21 to an average of 37 mmHg (ratio = 1.8), while blood gas pO_2 increased from 79 to 427 mmHg (ratio = 5.4; Duling and Berne, 1970). The reason for this effect is the non-linearity of the oxyhaemoglobin saturation curve, such that a large increase in arterial pO_2 produces only a modest increase in blood O_2 saturation. It is for this reason that simulations of O_2 transport suggest that improvements in tumour oxygenation will be modest with normobaric oxygen. Utilization of hyperbaric O_2 substantially increases the amount of oxygen dissolved in plasma and should further improve O_2 delivery (Brizel *et al.*, 1995; Secomb *et al.*, 1995).

Effects of carbogen and oxygen in changing arteriolar diameter and pO_2

The rationale for using carbogen in place of oxygen as a modifier of tumour oxygenation has centered around three assumptions (Rojas, 1992). First, it has been reported that carbogen blocks the vasoconstrictive effects of high oxygen content gases. Second, use of superphysiological levels of CO_2 should assist in the unloading of oxygen from haemoglobin. Third, carbogen has been reported to have positive chronotropic effects.

The results of this study are not exactly in concert with these assumptions. First, in both normal and tumour arterioles, we found that normobaric oxygen does not cause vasoconstriction. Variations in the vasoconstrictive properties of hyperoxia have been reported previously (Bertuglia *et al.*, 1991; Jackson, 1993), suggesting that this effect is tissue-dependent. Normobaric oxygen induces vasomotion in muscle arterioles, which is in concert with our results in both tumour and normal arterioles (Bertuglia *et al.*, 1991). The underlying mechanisms that control variation in arteriolar response to hyperoxia are not yet elucidated, but production of leucotrienes may be involved in some tissues (Jackson, 1993).

Second, we found that carbogen administration improved arteriolar pO_2 in both tumour and normal arterioles, but not to the same extent as oxygen breathing does. The observation of vasoconstriction in normal arterioles is opposite of what has been reported previously as a mechanism of action of this gas (Rojas, 1992). This may, in part, be due to the slight vasoconstrictive properties that carbogen seems to have in the normal tissue bed. The vasoconstrictive effects of carbogen may be the result of neuroregulatory responses to CO_2, at least in normal tissue. Prior studies of cat muscle under conditions of carbogen breathing showed a decrease in blood volume, cytochrome c oxidation level and muscle oxygen store, as measured with near infrared spectrophotometry. This effect could be blocked by pretreatment with bretylium, a sympatholytic agent (Hampson *et al.*, 1987). We have

shown that administration of hyperbaric carbogen yields less improvement in tumour oxygenation than hyperbaric oxygen. However, in animals pretreated with bretylium before administration of hyperbaric carbogen, the improvement in tumour oxygenation was equivalent to that achieved with hyperbaric oxygen (Brizel *et al.*, 1995). These results suggest that sympathomimetic effects also occur in conjunction with control over tumour blood flow, although the effects seem to be less prominent under normobaric conditions. The improvement in normal arteriolar pO_2 may occur, in spite of the vasoconstrictive effects because CO_2 facilitates unloading oxygen from haemoglobin (McGilvery and Goldstein, 1983). The addition of 5% CO_2 did not decrease blood pH, so the effect cannot be attributed to systemic acidosis-induced unloading of oxygen from haemoglobin (McGilvery and Goldstein, 1983).

Finally, the improvement in tumour arteriolar oxygenation was transient for both hyperoxic gases. The transient improvement in tumour oxygenation with carbogen has frequently been reported, including studies of human tumours, and the effect has been largely attributed to changes in blood flow (Falk *et al.*, 1992; Martin *et al.*, 1993; Siemann *et al.*, 1977; Rojas, 1992). It is possible that blood flow is decreasing during breathing with either gas, in spite of the lack of arteriolar vasoconstriction. Consequently, we are measuring the effects of these gases on tumour perfusion as well. However, it is difficult to comprehend a situation where net perfusion rates would decrease in the face of maintained or even dilated vascular tone, especially considering the positive effects that improved oxygenation would have on blood viscosity (Kavanagh *et al.*, 1993). An alternative explanation may be that the delivery of higher oxygen levels stimulates enhanced oxygen consumption, which then drives the pO_2 back toward baseline levels. There is evidence to suggest that tumours are capable of increasing oxygen consumption when they are given the opportunity (Kelleher and Vaupel, 1993).

An alternative explanation for the decrease in perivascular pO_2 during exposure to carbogen or oxygen is that high pO_2 levels could lead to electrode poisoning. This type of effect has been reported previously in studies of retinal oxygenation (Braun and Linsenmeier, 1995). However, the pO_2 necessary to induce this effect is much higher ($\geqslant$300 mmHg) than the measurements made in these arterioles. Secondly, when this effect occurs, the drop in measured pO_2 is much more abrupt than what was seen in these experiments. As an additional control we calibrated all electrodes both before and after each experiment. There was no drift in the calibration curves that would be indicative of poisoning.

Another potential explanation for the observed change in peri-arteriolar pO_2 is that the pO_2 changed in accordance with changes in arteriolar diameter (e.g. as a function of the distance between the electrode tip and the vessel wall). This argument is not valid, however, since the pO_2 begins to drop for both hyperoxic gases after 5 min of gas breathing, which is a time when arteriolar diameters are not changing.

A number of investigators have examined the effects of carbogen breathing on tumour oxygenation and radiosensitivity. The majority have shown positive effects with this gas, although the effectiveness has been variable (Chaplin *et al.*, 1993; Ono *et al.*, 1994; Siemann *et al.*, 1994; Song *et al.*, 1987; Thews *et al.*, 1995). In our hands, no improvement in oxygenation in flank R3230AC tumours has been observed, as assessed by polarographic techniques (Brizel *et al.*, 1995) Others have experienced similar results (Rockwell, 1985). Given the relative insensitivity of tumour oxygenation to increases in intravascular pO_2 in our model tumour (Secomb *et al.*, 1995), these results are not surprising.

Finally, it has been stated that the combination of carbogen and nicotinamide is effective because carbogen targets diffusion-limited hypoxia, while nicotinamide targets perfusion-limited hypoxia (Chaplin *et al.*, 1993; Kjellen *et al.*, 1991). While this provides a clear distinction of the effect of carbogen in this combination therapy, the real effects of this gas, and oxygen as well, are probably more complex than this. It is likely that gases that have vasoactive properties, such as carbogen, have some effect on perfusion-limited hypoxia as well.

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