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The effect of hypoxia and hyperoxia on nucleoside triphosphate/inorganic phosphate, pO_2 and radiation response in an experimental tumour model

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Summary This study has evaluated the effect of breathing 100% oxygen, carbogen and carbon monoxide (at 660 p.p.m.) on the bioenergetic and oxygenation status and the radiation response of 200-mm³ C3H mammary carcinomas grown in the feet of CDF mice. Bioenergetic status was assessed by ^{31}P magnetic resonance spectroscopy (MRS) using a 7-tesla spectrometer with both short (2 s) and long (6 s) pulse repetition times. Tumour partial pressure of oxygen (pO_2) was measured with an Eppendorf polarographic electrode; the oxygenation parameters were the median pO_2 and fraction of pO_2 values ≤ 2.5 mmHg. The radiation response was estimated using a tumour growth delay assay (time to grow three times treatment volume). Carbon monoxide breathing decreased tumour pO_2 and compromised the radiation response, but the β-nucleoside triphosphate (NTP)/P_i ratio was unchanged. Both carbogen and oxygen (100%) increased tumour pO_2 and β-NTP/P_i and enhanced the radiation response, the effects being similar under the two gassing conditions and dependent on the gas breathing time. Thus, in this tumour model, ^{31}P-MRS can detect hyperoxic changes, but because cells can remain metabolically active even at low oxygen tensions the β-NTP/P_i did not correlate with low tissue oxygenation. An analysis of variance showed that gas breathing time induced a significant systematic effect on β-NTP/P_i, the MRS pulse repetition time had a significant effect on β-NTP/P_i change under hypoxic but not under hyperoxic conditions and the type of gas that was inhaled had a significant effect on β-NTP/P_i.

Keywords: C3H mammary carcinoma; hyperoxia; hypoxia; ^{31}P-NMR spectroscopy; NTP/P_i; polarographic oxygen electrode; tumour oxygenation, radiation response

There is both experimental and clinical evidence that hypoxic tumour cells cause resistance to certain types of cancer therapy (Moulder and Rockwell, 1984; Teicher et al, 1981; Grau and Overgaard, 1988; Durand, 1991; Overgaard and Horsman, 1996). Considerable effort is now being made to identify those human tumours that contain hypoxic cells (for review see Stone et al, 1993, Raleigh et al, 1996). Direct estimates of tumour hypoxia by polarographic oxygen-sensitive electrodes have been shown to be clinically feasible, while new hypoxia marker assays such as detection of nitroimidazole labelling by the use of antibody techniques, ^{18}F-PET or ^{123}I-SPECT are currently being tested clinically. In addition, indirect estimates of tumour oxygenation have been reported, such as tumour blood perfusion measured by laser Doppler flowmeters, vascular staining techniques, functional magnetic resonance imaging (fMRI) or in vivo phosphorus magnetic resonance spectroscopy (^{31}P-MRS). Results obtained in human tumours using invasive oxygen electrodes show increasing evidence that the more hypoxic tumours are associated with a poorer treatment response to radiotherapy (Kolstad, 1968, Gatenby et al, 1988; Brizel et al, 1996; Höckel et al, 1996; Nordsmark et al, 1996). These clinical results warrant further experimental studies on how hypoxia causes treatment resistance; how tumour hypoxia can be modified in radiation therapy; and how sensitive the currently available techniques for detecting hypoxia are.

Previous studies in experimental tumours showed a significant positive correlation between ^{31}P-MRS energy measurements and oxygen status (Vaupel et al, 1989; Sostman et al, 1991), intracapillary oxyhaemoglobin saturation (Rofstad et al, 1988*a*), blood supply (Lyng et al, 1993) or radiobiological hypoxic fraction in three of four tumour lines (Rofstad et al, 1988*b*). In addition, a positive correlation was found between the fraction of radiobiological hypoxic cells and polarographic oxygen electrode measurements after manipulation of oxygen levels within tumours of a particular model and tumour size (Horsman et al, 1993). The ^{31}P-MRS energy status and the fraction of radiobiological hypoxic cells were compared under identical conditions and no correlation was found (Nordsmark et al, 1995). Moreover, no correlation was found in experimental studies that compared radiobiological hypoxia and oxygen electrode measurements (Horsman et al, 1995) or ^{31}P-MRS energy assessments (Rofstad et al, 1988*b*; Gerweck et al, 1995) across different tumour lines.

Thus, despite a considerable number of experimental studies, the usefulness of ^{31}P-MRS in detecting changes in tumour oxygenation and radiation response is not clear.

The aim of the present study was to determine the time effect of pretreatment inhalation of normobaric oxygen (100%), carbogen and carbon monoxide on ^{31}P-MRS energy assessment, tumour oxygenation status and radiation response. In the ^{31}P-MRS experiments we used a 6-s repetition time (TR_{6s}) and a 2-s repetition time (TR_{2s}) for comparison, in an attempt to minimize the T_1 dependency on the ^{31}P spectra. We assumed that the T_1 of inorganic

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phosphate (P_i) was longer than that of β-nucleoside triphosphate (β-NTP). Moreover, previous studies suggested that an increase in tumour oxygenation status would cause a decrease in T_1 of P_i because of the paramagnetic properties of oxygen (Okunieff et al, 1987, 1988). In addition, Olsen et al (1995), found that the T_1 of P_i decreased with increasing tumour size, possibly because of the release of paramagnetic metal ions during cell necrosis. Some of these earlier studies involved groups of tumours at different sizes as an additional variable to tumour pO_2 and ^{31}P-MRS, whereas in the present study tumours of identical size were used to eliminate confounding factors related to tumour growth.

MATERIALS AND METHODS

Tumour model

C3H mammary carcinomas were grown in the feet of 10- to 14-week-old CDF1/Bom (C3H/tif females crossed with DBA/2 males) male mice. The derivation and maintenance of the tumour has been described previously (Overgaard, 1980). Experiments were carried out when the tumour volume reached 200 mm^3 as determined by the formula $\pi/6 \times D_1 \times D_2 \times D_3$, where D_1, D_2 and D_3 represent the three orthogonal diameters. This tumour location was convenient as irradiation could be applied without the involvement of critical normal tissue in the field. Furthermore, it allowed all experiments to be performed in non-anaesthetized mice, restrained in a plastic jig with the tumour-bearing foot loosely taped to the jig to avoid occluding the blood supply.

Gas breathing

Mice were allowed to breathe either atmospheric air, 100% oxygen, carbogen (95% oxygen + 5% carbon dioxide) or air containing carbon monoxide at 660 p.p.m. (± 5%). The gas was administered through a nozzle placed over the restraining jig at a flow rate of 2.5 l min^{-1}. ^{31}P-MRS assessment was performed continuously for 64 min. For the first 16 min (8+8 min) there was no gas flow through the nozzle and the animals breathed air from within the magnet bore. This period served as the baseline control. An additional group of four animals (control) were studied without a nozzle; these animals breathed air from the magnet bore throughout. Electrode measurements of tumour oxygenation and tumour irradiations were carried out with pretreatment breathing times of 0, 5, 15, 30, 45 and 60 min, using a separate group of tumours at each time point, with the gas flow being maintained during the subsequent measurement or treatment period.

Radiation therapy

A conventional therapeutic X-ray machine (250 kV; 10 mA; 2 mm Al filter; 1.1 mm Cu half-layer; dose rate 2.3 Gy min^{-1}) was used. Only tumours were irradiated, the remainder of the animal being shielded by 1 cm of lead. To improve the dose homogeneity, tumours were immersed in a water bath with 5 cm of water between the X-ray source and the tumour. The tumour volume was measured five times a week after irradiation and treatment response was assessed by the time taken for a tumour to regrow to three times the treated volume (tumour growth time). Mice that died before the tumour reached three times the treatment volume were excluded from the analysis and any tumours controlled by the treatments were arbitrarily assigned a tumour growth time of 60 days.

^{31}P Magnetic resonance spectroscopy (^{31}P-MRS)

Assessment of tumour bioenergetic status was performed by ^{31}P-MRS using a 7-T Sisco spectrometer with an 18-cm horizontal bore. Phosphorus spectra were obtained from a homebuilt two-turn surface coil, 8 mm ID. The coil was placed over the tumour at approximately the same distance away from the foot in each case. Data were collected at 121.5 MHz with 4680 data points over a spectral width of 12 kHz in 8-min blocks alternating between 240 averages width TR_{2s} and 80 averages width TR_{6s}. Frequency-modulated (adiabatic) pulses (90° pulse over tumour volume by use of a 3-ms hyperbolic secant pulse) ensured a fairly homogeneous excitation of the whole tumour volume. Typically, the signal-to-noise ratio (S/N) for the highest peak was > 10 when measured in the tumour. The temperature around the mice was kept stable at 24°C by heated air flowing through the magnet bore during all measurements.

The background signal of ^{31}P from the underlying normal tissue of the foot was assessed using identical acquisition parameters in three animals. A 300-mm^3 spherical glass phantom containing 10 mM methylenediphosphonic acid was placed on the foot to simulate a tumour. Figure 1A shows examples of control spectra obtained from the dorsum of the mouse foot with and without a phantom. The phantom experiment resulted in a single peak equivalent to 20 mM phosphorus, with a resonance frequency of 20 p.p.m. corresponding to the symmetric molecule methylenediphosphonic acid. In the phantom experiment, the phosphocreatine (PCr) signal was very small. The experiment was then repeated without the phantom in all three animals and the only signal of any significance was PCr. In Figure 1B representative examples of spectra collected from individual tumours under different treatment conditions are shown. For the tumour spectra, the PCr signal was, in general, very low with a maximum S/N of 3. These results led us to conclude that any signal contribution from underlying normal tissue to the tumour spectrum of PCr and NTP was negligible.

Tumour oxygenation assessment

Tumour oxygenation status was assessed using polarographic oxygen electrodes (Eppendorf pO_2 Histograph, Germany). The method has been described in detail previously (Kallinowski et al, 1990). Briefly, the oxygen probe was inserted 1 mm into the tumour and automatically moved in a stepwise pattern, with a forward step of 0.7 mm followed by a backward step of 0.3 mm, thus giving 0.4 mm between each measurement. This procedure was repeated in four tracks, yielding 60 measurements per tumour.

Data analysis

The ^{31}P-MR spectra were analysed by time–domain fitting using VARPRO (van der Veen et al, 1988; van den Boogaart et al, 1995). The bioenergetic status was defined as the ratio of β-nucleoside triphosphate to inorganic phosphate (β-NTP/P_i) obtained from each tumour, as no standard reference was used to enable comparison of individual peak intensities between tumours. PCr was of very little significance in this tumour. Therefore, the β-NTP/P_i was the ratio that gave an estimate of the proportion of high and low metabolic energy compounds. The effect of gas breathing on tumour bioenergetic status was evaluated as the relative change in the β-NTP/P_i ratio, which was given by the following equation:

$$\Delta\ \beta\text{-NTP/P}_i = (\beta\text{-NTP/P}_{i\ \text{time x}} - \beta\text{-NTP/P}_{i\ \text{time 0}})/\beta\text{-NTP/P}_{i\ \text{time 0}}$$

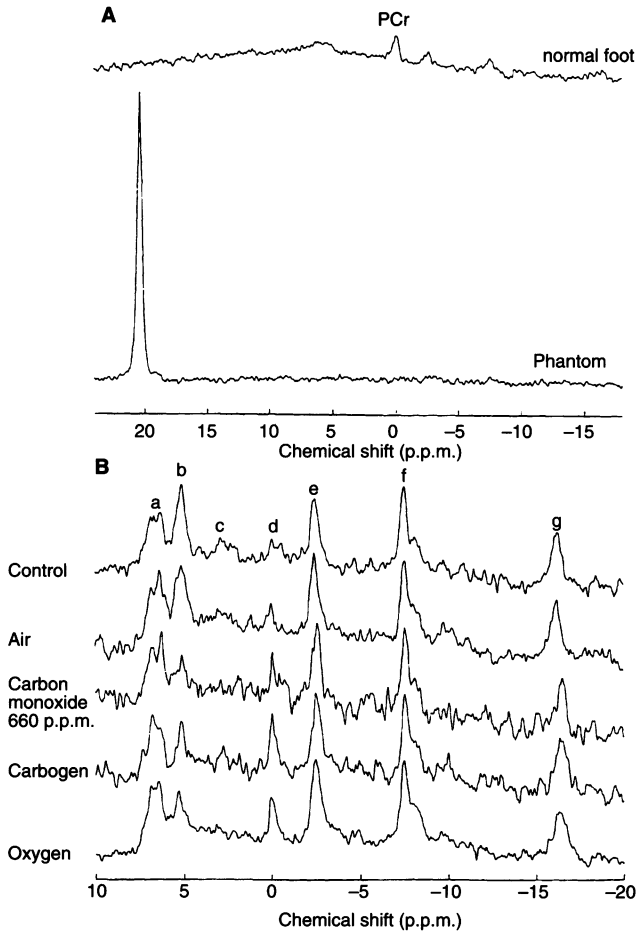

Figure 1 (**A**) The upper spectrum is a representative example of background signal of the ^{31}P from the underlying normal tissue of a mouse foot. PCr, phosphocreatine. The lower spectrum is obtained from a phantom, which contains methylendiphosphonic acid 10 mM, that was placed on the foot to simulate a tumour. (**B**) Representative examples of ^{31}P spectra obtained from individual tumours under different treatment conditions. Peak assignments are a, phosphomonoesters; b, inorganic phosphate; c, phosphodiesters; d, phosphocreatine; e, γ-nucleoside triphosphates and β-nucleoside diphosphates; f, α-nucleoside triphosphates and α-nucleoside diphosphates; g, β-nucleoside triphosphates

From the raw pO_2 data obtained using the polarographic electrode measurements, two parameters were derived: the median tumour pO_2 and the fraction of pO_2 values ≤ 2.5 mmHg. The latter value is an estimate of the relative frequency of the measurements below the level of radiobiological hypoxia.

The experimental data from each group of animals were summarized as means, standard error of the mean and standard deviation. Results were compared using the Students *t*-test. A mixed model analysis of variance was performed using the BMDP statistical program (Dixon, 1990). A significance level of 5% was used.

RESULTS

Bioenergetic status

The spectra in Figure 1 are representative of the spectra of the tumours examined under the different conditions. When analysing baseline β-NTP/P_i levels of individual tumours a large intertumour variability was found, as seen from Figure 2A. In all gas-breathing experiments, the tumour bioenergetic status was assessed in blocks alternating between TR_{2s} (240 averages) and TR_{6s} (80 averages). The purpose of using 6 s as the repetition time was to minimize any T_1 effect on the signal intensity and still allow a reasonable resolution in time, which would not be possible under the ideal conditions using fully relaxed measurements. The results shown in Figure 2A represent the initial two blocks (time 0–16 min) obtained under baseline conditions before any treatment. As represented in Figure 2A, there was a significant correlation between the β-NTP/P_i ratio for the TR_{2s} and the TR_{6s} under normal conditions ($r^2 = 0.329$; $P < 0.001$), but the β-NTP/P_i ratios obtained with TR_{2s} were significantly higher than with TR_{6s} ($P < 0.0001$).

Figure 2B illustrates the effect of T_1 on signal saturation for TR_{2s} and TR_{6s} as predicted from the relationship:

$$\text{maximal signal proportion} = 1 - e^{-TR/T1}$$

By using TR_{6s}, the relative signal intensity will change by 11% per second change in T_1, compared with 20% per second T_1 change using TR_{2s}. Results obtained in the present study showed that the baseline β-NTP/P_i ratios obtained during TR_{6s} were lower than the values of TR_{2s}, which suggests that the P_i signal intensity was higher at the longer repetition time. This is in accordance with a T_1

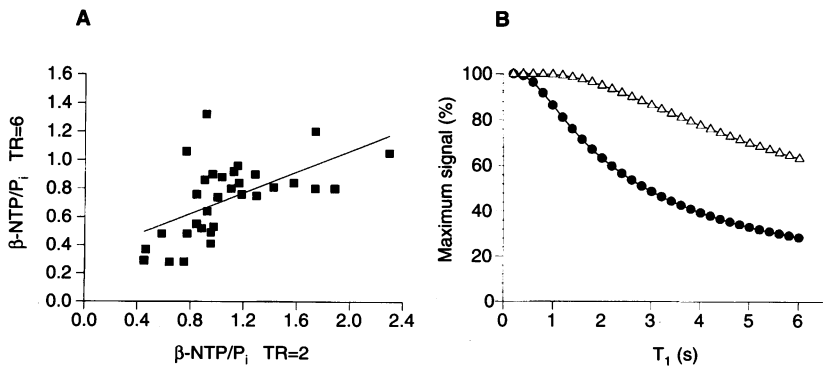

Figure 2 (**A**) The relationship between β-NTP/P_i using 2- and 6-s pulse intervals. Each symbol represents the β-NTP/P_i energy status from individual untreated mice using the 16-min period before treatment. $n = 31$. (**B**) The maximal signal intensity of TR_{2s} (●) and TR_{6s} (△) as a function of T_1 relaxation time

Table 1 Signal intensities of P_i and β-NTP during inhalation of high- and low-oxygen content gas mixtures

		Gas-breathing time (min)				
Gas mixture	***n***	**0**	**8**	**24**	**40**	**56**
P_i (TR_{6s}/TR_{2s})						
Control	4	1.8	1.9	2.0	2.0	2.0
Atmospheric air flow	9	2.3	1.8	2.2	2.0	1.9
Carbogen	7	1.8	1.8	2.0	2.2	1.9
Oxygen	6	2.4	2.0	2.0	2.0	2.2
Carbon monoxide (660 p.p.m.)	5	1.9	1.9	2.1	1.9	1.9
β-NTP (TR_{6s}/TR_{2s})						
Control	4	1.4	1.4	1.4	1.3	1.3
Atmospheric air flow	9	1.3	1.3	1.3	1.4	1.3
Carbogen	7	1.3	1.2	1.4	1.5	1.6
Oxygen	6	1.3	1.3	1.3	1.3	1.4
Carbon monoxide (660 p.p.m.)	5	1.2	1.5	1.4	1.3	1.4

n, number of tumours; TR, repetition time; P_i, inorganic phosphate; *β*-NTP, *β* nucleoside triphosphate. All peak intensities at TR_{2s} were measured at the time points listed in the table. Values at TR_{6s} were calculated from the intensity 8 min before to 8 min after time 8, 24, 40 and 56 min.

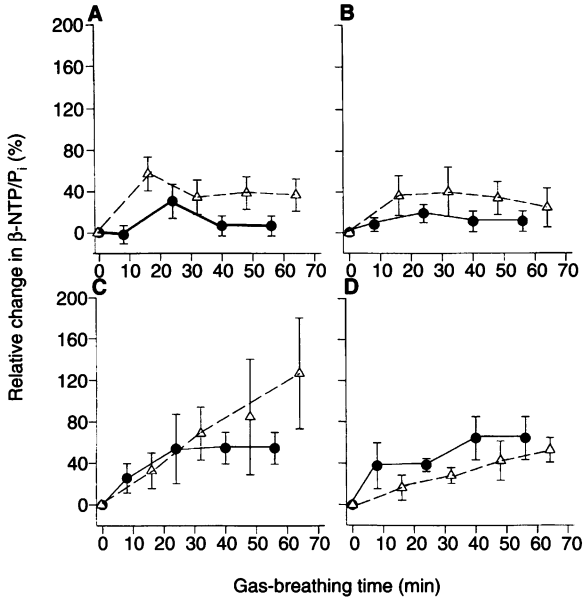

Figure 3 The relative change in β-NTP/P_i = Δ β-NTP/P_i = (β-NTP/$P_{i\ time\ x}$ – β-NTP/$P_{i\ time\ 0}$)/β-NTP/$P_{i\ time\ 0}$ measured during inhalation of atmospheric air (a), carbon monoxide (b), carbogen (c) and oxygen 100% (d). ● = 2 s repetition time, △ = 6 s repetition time. Each point represents the average from groups of 4–9 mice. Error = standard error of the mean

value of 4.2 s for P_i and 1.3 s for β-NTP found in a C3H fibrosarcoma at 8.5 T (Vaupel et al, 1990). Despite an increase in the TR_{6s}/TR_{2s} ratio of the signal intensity for both P_i and β-NTP, as shown in Table 1, there was no dependence of gas breathing time or type of gas inhaled on the relative signal intensity of P_i and β-NTP. This suggests that any T_1 effect was minimized by using TR_{6s} in the present study.

The relative change in bioenergetic status

Because of the large intertumour variability, each tumour was used as its own control and the relative change in bioenergetic status (Δ β-NTP/P_i = (β-NTP/$P_{i\ time\ x}$ – β-NTP/$P_{i\ time\ 0}$)/β-NTP/$P_{i\ time\ 0}$) was chosen as the endpoint when modifying the O_2 availability to the tumour. Figure 3 shows the relative change in β-NTP/P_i as a function of time under different gas breathing conditions for TR_{2s} and TR_{6s}. Atmospheric air flow at 16 min (TR_{6s}) caused a rise in the Δ β-NTP/P_i ($P = 0.01$) followed by a decrease to a level that was not significantly different from baseline ($P = 0.08$). In the TR_{2s} measurement of atmospheric air flow there was a similar trend towards an increase followed by a decrease in the bioenergetic status. The reason that atmospheric air flow induced this relative increase in the β-NTP/P_i is not clear. One explanation could be that the air flow caused an initial decrease in tumour temperature when introduced inside the magnet, despite the compensatory heating system. This may have led to a decrease in cellular oxygen consumption and subsequently an increase in the β-NTP/P_i energy status. Another explanation could be that mice were initially

Table 2 The effect of hypo- and hyperoxic gas types on *β*-NTP/P_i at a range of breathing times

		Relative change in β-NTP/P_i (%) at gas-breathing time (min)							
Gas type	***n***	**8**	**16**	**24**	**32**	**40**	**48**	**56**	**64**
TR_{2s}									
Control	4	3		1		1		6	
Atmospheric air flow	9	–2		30		9		7	
Carbogen	7	26		54		81*		55*	
Oxygen	6	38		39		48*		64*	
Carbon monoxide (660 p.p.m.)	10	8		19		28		11	
TR_{6s}									
Control	4		–6		–11		–9		–8
Atmospheric air flow	9		57		34		39		37
Carbogen	7		33		69		85		127
Oxygen	6		17		28		43		53
Carbon monoxide (660 p.p.m.)	5		36		40		34		24

Each value is the average of the relative change in β-NTP/P_i of animals breathing atmospheric air flow compared with that of carbogen, 100% oxygen and carbon monoxide (660 p.p.m.) using Student's *t*-test. *, Significant ($P < 0.05$). *n*, number of tumours. TR, repetition time.

Table 3 A mixed model analysis of variance

Test groups	n	P-value for variables		
		Gas type	Breathing time	Repetition time (TR)
All four gas types*	32	0.008	< 0.001	NS**
Oxygen + carbogen	13	NS	< 0.001	NS
Oxygen	6		0.001	NS
Carbogen	7		< 0.001	NS
Carbon monoxide (660 p.p.m.)	5 (+5***)	NS	0.03	
Atmospheric air	9		0.002	NS

The probability that any of the variables gas type, gas breathing time and repetition time have an effect on the relative change in β-NTP/P_i. NS, not significant. *Atmospheric air, carbon monoxide, oxygen 100% and carbogen. **27 animals. ***^{31}P-MRS data available for TR_{2s} alone.

stressed by being placed in the magnet or that oxygen availability for the mice in the straining jig was improved by switching on the air flow. However, there was no apparent effect on tumour oxygenation status or its response to radiation.

Breathing carbon monoxide (660 p.p.m.) induced relative changes in the β-NTP/P_i ratio similar to those of atmospheric air. Both carbogen and oxygen 100% caused a continuous increase in the Δ β-NTP/P_i using TR_{6s}. But, when using TR_{2s} and inhaling oxygen (100%) or carbogen, the Δ β-NTP/P_i showed an initial increase at 8 and 24 min, respectively, and then seemed to reach a plateau.

Table 2 shows results of the relative change in the β-NTP/P_i during inhalation of atmospheric air flow compared with that of carbogen, 100% oxygen and carbon monoxide (660 p.p.m.) respectively. Atmospheric air flow was chosen as the reference, and not the control group left in the scanner without air flow because of an initial increase in the relative change in the β-NTP/P_i. There was a time-dependent increase in the Δ β-NTP/P_i for both oxygen and carbogen using TR_{6s}, but this improvement was not significantly different from that of animals breathing atmospheric air. However, when using TR_{2s} and breathing carbogen or oxygen (100%) the Δ β-NTP/P_i was significantly higher than that of controls at intermediate breathing times of 40 and 56 min.

The influence of inhalation gas type, gas breathing time and the MR parameter TR on the relative change in the β-NTP/P_i was tested further in a mixed model of variance. These results are summarized in Table 3. The null hypothesis was that the relative change in the β-NTP/P_i was independent of the following variables – gas type, breathing time and repetition time – and the analysis was performed in test groups that involved either all four gas types, the two hyperoxic gas mixtures of oxygen (100%) and carbogen, or by testing the effect of breathing time and repetition time of each gas type alone. Variance analysis showed that in all situations, apart from carbon monoxide, the gas-breathing time induced a significant systematic effect on Δ β-NTP/P_i. The analysis also showed that repetition time had a significant effect on the Δ β-NTP/P_i during carbon monoxide breathing, but no effect when analysed for the remaining test conditions. When all four gas types were considered the gas type had a significant effect on Δ β-NTP/P_i, but there was no systematic difference in Δ β-NTP/P_i induced by oxygen (100%) compared with carbogen.

Tumour oxygenation

Figure 4 shows the time dependence of breathing low- or high-oxygen gas mixtures on the average of the tumour median pO_2 and the average of the fraction of pO_2 values ≤ 2.5 mmHg. Inhalation of atmospheric air had no impact on the oxygenation status. Carbon monoxide (660 p.p.m.) produced a continuous and significant decrease in the median tumour pO_2 ($P = 0.01$ at 45 min) and a significant increase in the fraction of pO_2 values ≤ 2.5 mmHg relative to initial baseline levels. Both 100% oxygen and carbogen gas breathing improved the median tumour pO_2 significantly within 5 min breathing time and reduced the fraction of low readings significantly by 5 and 15 min respectively.

Radiation response

The influence of varying the preirradiation breathing time of low or high oxygen content gas mixtures on the radiation response of this C3H tumour to a single dose of 15 Gy X-rays, is shown in Figure 5. Inhalation of atmospheric air had no effect on tumour growth delay (Figure 5A). Carbon monoxide (660 p.p.m.) compromised the response to radiation, but the effect was not as great as the delay in tumour growth achieved by total occlusion of the

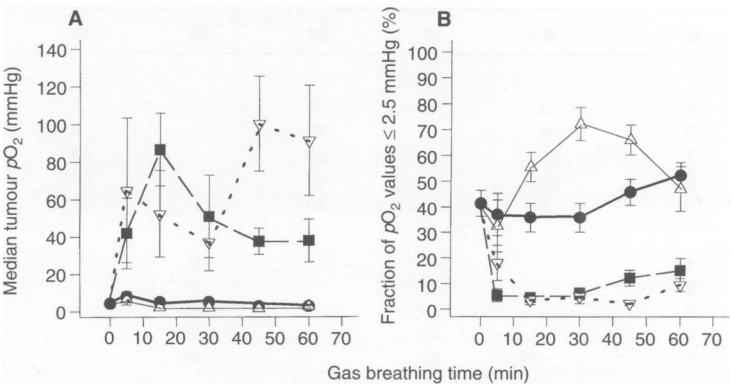

Figure 4 The time dependency of the average of the median tumour pO_2 (**A**) and the fraction of pO_2 values ≤ 2.5 mmHg (**B**), while breathing (●) atmospheric air, (△) carbon monoxide (660 p.p.m), (■) carbogen and (▽) 100% oxygen. Each point represents the average of measurements from six or seven mice. Error, standard error of the mean

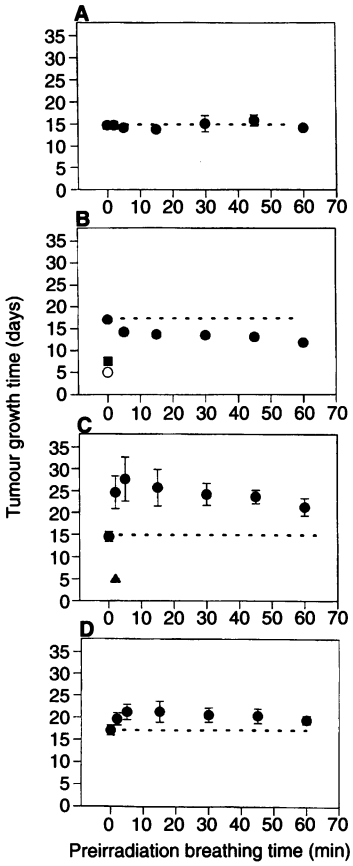

Figure 5 The effect of preirradiation breathing time of atmospheric air, A; carbon monoxide 660 p.p.m., B; carbogen, C; and pure oxygen, d; on tumour radiation response. Mice were allowed to breathe the different gas mixtures for varying time periods before and during local tumour irradiation and the time taken for tumours to grow to three times their treatment volume was recorded. Between seven and 12 mice were used per treatment group and between three and seven in controls. Symbols represent the average tumour growth time of untreated controls (○), carbogen alone (▲), clamping by tightening a rubber tube around the tumour-bearing leg for 5–20 min before and during irradiation to occlude the blood supply (■), radiation alone (- - - -), radiation + gas breathing (●). Data for the effect of 100% oxygen alone were not available. Error, standard error of the mean

blood supply (Figure 5B), whereas both carbogen (Figure 5C) and 100% oxygen (Figure 5D) enhanced radiation damage.

DISCUSSION

Tumour oxygenation and radiation response

In the present study, both oxygen (100%) and carbogen breathing improved the oxygenation status and enhanced the radiation response of this C3H mammary carcinoma. These results are consistent with a number of experimental studies (Siemann et al, 1977; Grau et al, 1992; Chaplin et al, 1993; Horsman et al, 1994; Brizel et al, 1995). The results of the clinical trials that tested the effect of normobaric and hyperbaric oxygen, and carbogen breathing were conflicting (Overgaard and Horsman 1996), although inhalation of carbogen has been reported to improve the oxygenation status in human tumours (Falk et al, 1992; Martin et al, 1993). The lack of success in some of the earlier trials may partly be explained by the fact that gas inhalation was often interrupted or not performed during the radiation treatment (Rubin et al, 1979). Our results, and those of others (Siemann et al, 1977; Chaplin et al, 1993), clearly show that preirradiation breathing time of 100% oxygen and carbogen, and continuous gas breathing during irradiation affect the tumour pO_2 and the enhancement of radiation damage. However, inhalation of these hyperoxic gas mixtures did not eradicate hypoxia in all cases.

The current study showed that carbon monoxide (660 p.p.m.) breathing caused radiation resistance. This was demonstrated previously by Grau (1994) for local tumour control after single dose and fractionated irradiation in which carbon monoxide breathing caused elevated HbCO levels that led to increased tumour hypoxia and radiation resistance. But the radiation modification from breathing carbon monoxide (660 p.p.m.) was not as severe as when occluding the blood supply by clamping.

The usefulness of ^{31}P-MRS in detecting changes in tumour pO_2 and radiation response

The current study showed that a significant reduction in tumour oxygenation induced by carbon monoxide inhalation had no effect on the bioenergetic status of the tumours. These results suggest that ^{31}P-MRS energy measurement does not correlate with levels of low tissue oxygenation in this tumour model. Okunieff (1987) reported a decrease in the PCr/P_i ratio following inhalation of 10% oxygen and 90% nitrogen in the FSaII murine fibrosarcoma but their study is not strictly comparable to the present one because tumours of different sizes were compared, and because a different parameter for bioenergetic status was used. However, Sostman et al (1991) detected a decrease in rhabdomyosarcomas of equal size in non-anesthetized mice that were breathing 5% oxygen and 95% nitrogen. Thus, the ability of ^{31}P-MRS to detect changes in low tumour pO_2 depends on the ability of the tumour to produce high-energy phosphates by anaerobic glycolysis.

Although we found that the induction of hypoxia had no impact on the relative change in β-NTP/P_i, exposure to both 100% oxygen and carbogen was followed by an increase in the relative change in β-NTP/P_i as a function of gas-breathing time. This finding is in agreement with the results from other studies (Okunieff et al, 1987; Sostman et al, 1991; Gerweck et al, 1993).

Constant β-NTP/P in hypoxic tumours

In a previous study, using the C3H mouse mammary carcinoma, Grau (1994) demonstrated that breathing carbon monoxide caused a time- and dose-dependent formation of carboxyhaemoglobin and a reduction in blood flow. At 660 p.p.m. the carboxyhaemoglobin had increased from a control value of 2% to 45%, whereas blood flow was only at 20% of that found in control tumours. The low tumour oxygenation is likely to be a consequence of both the increase in carboxyhaemoglobin and the reduction in blood flow, whereas the intact energy metabolism is most probably explained by a sufficient supply of glucose and/or other nutrients for anaerobic glycolysis even under these conditions. This hypothesis is supported by other studies showing that the tumour bioenergetic status was dependent on alterations in blood flow, oxygen availability (Vaupel et al, 1994a) and nutritional resources to sustain the energy metabolism (Gerweck et al, 1993). Using an in vitro assay and a different tumour model, it was demonstrated that the energy status was stable during oxygen deprivation but with the availability of sufficient glucose (Gerweck et al, 1993). Moreover, the inhibition of glycolysis by 2-deoxyglucose and insulin caused a decrease in the ATP/P_i ratio of an experimental sarcoma rat tumour model (Karczmar et al, 1992).

Repetition time and assessment of bioenergetic status

The present study showed that a 6-s pulse interval (compared with 2 s) caused a higher signal intensity increase of P_i than of β-NTP, which resulted in a reduction in the β-NTP/P_i ratio of about 1.5, but there was no additional increase in the P_i signal intensity after breathing oxygen 100% or carbogen. Therefore, the improvement in P_i intensity by using TR_{6s} is most probably because the T_1 effect of P_i was minimized whereas the suggested paramagnetic effect of oxygen on P_i was less important in this tumour model. T_1 of P_i and β-NTP was not measured in the current study, but Okunieff (1988) reported T_1 values of 5.93 s for P_i of anoxic tumours in mice that had been dead for 60 min. Moreover, the T_1 of phosphorus resonances was found to differ significantly between tumour models and to be dependent on tumour volume and on the oxygenation status of the tumour (Okunieff et al, 1986, 1987, 1988; Olsen et al, 1994, 1995). Finally, in vitro experiments have documented that metallic ions, probably present in the debris of necrotic regions are also likely to reduce T_1 of P_i (McCain, 1987).

Conclusion

In conclusion, inhalation of carbon monoxide was associated with enhanced radiation resistance, a decrease in tumour oxygenation and unchanged bioenergetic status expressed by the β-NTP/P_i ratio. These results suggest that in this tumour model cells can remain metabolically active even at low oxygen tensions, which makes the ability of ^{31}P-MRS to detect changes in low tumour pO_2 dependent on the potential of the tumour to produce high energy phosphates by anaerobic glycolysis. Induction of hyperoxia by breathing carbogen or oxygen (100%) was followed by an increase in β-NTP/P_i, tumour pO_2 and an enhancement of radiation response as a function of gas-breathing time.

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