Int. J. Radiation Oncology Biol. Phys., Vol. 55, No. 4, pp. 1066–1073, 2003

0360-3016/03/$–see front matter

ELSEVIER

doi:10.1016/S0360-3016(02)04505-4

BIOLOGY CONTRIBUTION

NITRIC OXIDE–MEDIATED INCREASE IN TUMOR BLOOD FLOW AND OXYGENATION OF TUMORS IMPLANTED IN MUSCLES STIMULATED BY ELECTRIC PULSES

BÉNÉDICTE F. JORDAN, M.S.,*† PIERRE SONVEAUX, M.S.,‡ OLIVIER FERON, PH.D.,‡
VINCENT GRÉGOIRE, PH.D.,§ NELSON BEGHEIN, B.SC.,*† AND BERNARD GALLEZ, PH.D.*†

*Laboratory of Medicinal Chemistry and Radiopharmacy, †Laboratory of Biomedical Magnetic Resonance, ‡Laboratory of Pharmacotherapy, and the §Radiobiology and Radioprotection Unit, Université Catholique de Louvain, Brussels, Belgium

Purpose: Oxygen deficiency in tumors reduces the efficacy of nonsurgical treatment modalities. We tested the hypothesis that electrical stimulation of the sciatic nerve could modify the oxygenation status and the blood flow of tumors implanted in the thigh of mice.

Methods and Materials: The sciatic nerve was electrically stimulated at 5 Hz. Local transplantable liver tumor (TLT) and fibrosarcoma (FSaII) tumor oxygen pressure (pO_2) and perfusion measurements were carried out using electron paramagnetic resonance (EPR) oximetry and the OxyLite/OxyFlo technique. The radiosensitizing effect of the protocol was assessed by irradiating FSaII tumors with X-rays.

Results: Tumor pO_2 increased from ~3 mm Hg to ~8 mm Hg, and relative tumor blood flow was increased by 241% and 162% for TLT and FSaII tumor models, respectively. The effect on the tumor oxygenation was inhibited by a nitric oxide synthase (NOS) inhibitor, and an increase in the tumor nitric oxide (NO) content was observed using EPR spin-trapping. The tumor oxygen consumption rate was decreased after the stimulation protocol. In addition, the electrical stimulation of the host tissue increased regrowth delays by a factor of 1.65.

Conclusions: This increase in tumor oxygenation is due to the temporary increase in tumor blood flow, but particularly to a decrease in the tumor oxygen consumption rate (inhibition of respiration) that is mediated by a local production of NO during the protocol. Those tumor hemodynamic changes resulted in a radiosensitizing effect. © 2003 Elsevier Science Inc.

Tumor, Oxygenation, Perfusion, Nitric oxide, Electric pulse.

INTRODUCTION

Tumor oxygenation and blood flow are of fundamental importance to many forms of cancer therapy. Oxygen deficiency in tumors reduces the efficacy of nonsurgical treatment modalities such as conventional radiotherapy and chemotherapy. This oxygen deficiency is caused by an insufficient oxygen supply as a result of inadequate tumor perfusion (diffusion-limited hypoxia) and fluctuations in red cell flux (acute hypoxia) (1–5). Therefore, blood flow modifiers are investigated for their potential benefit in terms of treatment efficacy (6). The use of vasoactive agents (7) or carbogen breathing (8, 9) are also evaluated for their potential therapeutic interest. Other interventions are aimed at radiosensitizing hypoxic cells (10). However, only a few studies have focused on the role of the physiologic conditions of the host tissues.

We aimed to test the hypothesis that the electrical stimulation of the host tissue could modify the oxygenation status and the blood flow of tumors implanted in the thigh of mice. Skeletal muscle blood flow is increased in response to physical exercise in order to supply additional oxygen to meet the muscle metabolic demand (11, 12). Intermuscular and intramuscular perfusion differences were measured during exercise by [^{15}O]-H_2O positron emission tomography imaging in healthy subjects (13). Exercise training elevates nitric oxide (NO) bioavailability through a variety of mechanisms, including increased nitric oxide synthase (NOS) enzyme expression and activity (14). Koller *et al.* found that removal of endothelium eliminated flow-dependent dilation and that inhibition of NO synthesis significantly reduced the dilation to increases in flow in rat skeletal muscle arterioles (11). Gilligan *et al.* also showed a reduced exercise-induced

Reprint requests to: Prof. Bernard Gallez, Laboratory of Medicinal Chemistry and Radiopharmacy, Avenue Mounier 73.40, B-1200 Brussels, Belgium. Tel: 32 2 7642792; Fax: 32 2 7642790; E-mail: Gallez@cmfa.ucl.ac.be

Supported by the Belgian National Fund for Scientific Research (Grant no. 3.4560.00) and the Fonds Joseph Maisin. Bénédicte Jordan and Pierre Sonveaux are research assistants of the Belgian National Fund for Scientific Research. Olivier Feron is Research Associate of the Belgian Fund for Scientific Research.

Acknowledgments—The authors wish to thank Philippe Gailly for providing the electric stimulator.

Received Apr 22, 2002, and in revised form Oct 1, 2002. Accepted for publication Nov 20, 2002.

vasodilation in the human forearm after inhibition of NOS with N-monomethyl-L-arginine (L-NMMA) (15). Roberts *et al.* finally demonstrated that acute exercise increased both neuronal and endothelial NOS activity in rats (16).

We wanted to know if the oxygenation and blood flow could increase in tumors implanted in muscles in response to electrical stimulation of the host tissue. We monitored the tumor oxygen pressure (pO_2) during and after a 15-min stimulation protocol on two different tumor models. This effect was evaluated using low-frequency electron paramagnetic resonance (EPR) oximetry and the OxyLite technique. Modification of the tumor blood flow was estimated by the OxyFlo technique. These methods are adequate to assess modifications of local pO_2 as well as perfusion of the tumors (7, 17–19). In addition, the tumor cells' oxygen consumption rate was measured *ex vivo* using EPR spectroscopy (20). Finally, to establish the therapeutic relevance of this approach, the radiation response of fibrosarcoma (FSaII) tumors that were electrically stimulated was assessed by local irradiation with 16 Gy of X-rays. We further identified the likely underlying mechanism as a NO pathway. For this purpose, we quantified the cyclic guanosine monophosphate (cGMP) tumor content and detected an increase in tumor NO production by EPR spin-trapping during electrical stimulation of the host tissue.

METHODS AND MATERIALS

Animal tumor models

Two different tumor models were implanted in the thigh of mice: a transplantable liver tumor model (TLT) (21) on NMRI mice and the syngeneic FSaII tumor model (22) on C3H mice. The measurements were carried out when the tumor diameter was 8 ± 1 mm. For each tumor, transversal and anteroposterior measurements were obtained. An average tumor diameter was then calculated.

Experimental protocol

Anesthesia was first induced by an intraperitoneal injection of ketamine (80 mg/kg)/xylazine (8 mg/kg) and maintained with ketamine alone (30 mg/kg). Mice were maintained at 37°C using an homeothermic blanket. Platinum electrodes were placed around the right sciatic nerve after removing of the skin and exposure of the nerve. A 15-min exercise protocol was achieved by electrical stimulation at 5 Hz, with 0.2-ms duration pulses (23). When used, the NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME, Sigma, Steinheim, Germany) was injected intraperitoneally at 15 μmol.kg^{-1}, 1 h before the exercise protocol (24).

pO_2 and blood flow measurements

Local tumor oxygenation measurements were carried out using two independent techniques: EPR oximetry and a fiber-optic device, OxyLite. We used the OxyFlo system to assess the blood flow inside the tumor.

EPR oximetry. EPR spectra were recorded using an EPR spectrometer (Magnettech, Germany) with a low-frequency microwave bridge operating at 1.2 GHz and extended loop resonator. Charcoal (Charcoal wood powder, CX0670-1, EM Science, Gibbstown, NJ) was used as the oxygen-sensitive probe in all experiments. Calibration curves were made by measuring the EPR line width as a function of the pO_2. For this purpose, the charcoal was suspended in a tumor homogenate and EPR spectra were obtained on a Bruker EMX EPR spectrometer (9 GHz) between 0 and 21% O_2. Nitrogen and air were mixed in an Aalborg gas mixer (Monsey, NY), and the oxygen content was analyzed using a Servomex oxygen analyzer OA540 (25). Mice were injected in the center of the tumor (8-mm diameter) using the suspension of charcoal (100 mg/mL, 50 μL injected, 1–25 microns particle size). The EPR measurements were started 2 days after the injection. The tumor under study was placed in the center of the extended loop resonator which had a sensitive volume extending 1 cm into the tumor mass, using a protocol previously described (7, 26).

Oxylite/Oxyflo technique. We used the Oxylite in conjunction with Oxyflo (Oxford Optronix, Oxford, UK) for simultaneously and continuously monitoring tissue blood flow, oxygenation, and temperature at the same location (18, 19). Fiber-optic microprobes combining a laser Doppler system, an oxygen-sensor that is based on the fluorescence quenching of a ruthenium dye, and a thermocouple were inserted both into the tumor and in the muscle. Probes were inserted using a 26G needle to make a track inside the tissue. Back scattering measurements were used to validate the absence of movement artifact influence on pO_2 and flow measurements. A baseline of 15 min of stable readings was obtained before starting the stimulation protocol. Data were collected continuously at a sampling frequency of 20 Hz, before, during, and after electrical stimulation. Oxylite pO_2 measurements are single point measurements; the volume sampled is confined to the sensor tip (230 μm diameter).

Oxygen consumption rate evaluation

The method developed by James *et al.* (20) was used. All spectra were recorded on a Bruker EMX EPR spectrometer operating at 9 GHz. The exercise protocol was first performed *in vivo*. Thirty minutes after the end of the sciatic nerve stimulation, tumors were excised, trypsinized for 30 min, and cell viability was determined as previously reported. Cells (2×10^7/mL) were suspended in 10% dextran in complete medium. A neutral nitroxide, ^{15}N PDT (4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-^{15}N-1-oxyl, CDN isotopes, Quebec, Canada) at 0.2 mM, was added to 100-μL aliquots of tumor cells that were then drawn into glass capillary tubes. The probe (0.2 mM in 20% dextran in complete medium) was calibrated at various [O_2] between 100% nitrogen and air so that the linewidth measurements could be related to [O_2] at any value. The sealed tubes were placed into quartz EPR tubes, and samples were maintained at 37°C. As the resulting linewidth reports on [O_2], oxygen consumption rates were obtained by measuring the [O_2] in the closed tube over time and finding the slope of the resulting linear plot.

Irradiation and tumor regrowth delay assay

The tumor-bearing leg was locally irradiated with 16 Gy of 250-kV X-rays (RT 250, Philips Medical Systems, Brussels, Belgium). Mice were anesthetized, and the tumor was centered in a 3-cm-diameter circular irradiation field. When tumors reached 8.0 ± 0.5 mm in diameter, the mice were randomly assigned to a treatment group and irradiated. After treatment, tumors were measured every day until they reached a diameter of 15 mm, at which time the mice were killed. A linear fit could be obtained between 8 and 15 mm, which allowed us to determine the time to reach a particular size for each mouse.

cGMP quantification

Electrically stimulated or control FSaII-bearing mice were killed, and tumors were homogenized in cold 6% (wt/vol) trichloroacetic acid. Samples were then centrifuged at 2000 g for 15 min at 4°C. The supernatant was washed 4 times with 5 volumes of water-saturated diethyl ether. The aqueous extract remaining was dried under a stream of nitrogen at 60°C, and the dried extract was dissolved in a 0.05 M sodium acetate buffer before analysis. The cGMP content was then determined by the cGMP enzyme immunoassay kit from Biotrak (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany).

Ex vivo NO spin-trapping experiment

The detection of NO produced in FSaII tumors was conducted using the NO trapping method with the water-soluble iron–MGD complex (iron–*N*-methyl-$_D$-glucamine dithiocarbamate). MGD and iron form a paramagnetic complex with NO (NO-$Fe^{2+}(MGD)_2$), which gives a characteristic triplet EPR spectrum (27, 28). The sodium salt of MGD was from Alexis Biochemicals (Brussels, Belgium). For the control group, 200 μL of a freshly prepared solution of iron sulfate (20 mM) and MGD (100 mM) was administered intravenously to anesthetized tumor-bearing mice. Thirty minutes later, mice were killed and the tumor was taken, homogenized, and immediately frozen in liquid nitrogen. In this 30-min period, the experimental group was electrically stimulated during the last 15 min and mice were killed immediately after the end of the stimulation period (to use the same timing as for the control group). The EPR spectra were recorded at 77 K on a Bruker EMX EPR spectrometer operating at 9.4 GHz. Typical spectrometer conditions were as follows: incident microwave power: 40 mW; modulation amplitude: 2 Gauss; sweep width: 150 Gauss; time constant: 5, 12 ms; 200 scans accumulation.

RESULTS

Effect of the electrical stimulation protocol on tumor oxygenation

EPR oximetry relies on the oxygen-dependent broadening of the EPR linewidth of a paramagnetic oxygen sensor implanted in the tumor (7, 17). The fiber-optic device, OxyLite, allows pO_2 measurement that is based on the oxygen-quenched lifetime of a luminescent ruthenium dye (18, 19). The two techniques we used are intended for continuous measurement of the local pO_2 without altering the local oxygen concentration, and allow a real-time study of the oxygen fluctuations in tissues. The stimulation of the sciatic nerve modified tumor pO_2 for both models. Oxygenation increased during exercise and was maintained at least 30 min after the end of the protocol (Fig. 1 and Table 1). Both the pO_2 reached during stimulation and the time course of increasing pO_2 were quite variable from one tumor to another. The basal tumor pO_2 was very low (around 3 mm Hg). The final pO_2 that we observed during exercise (around 8 mm Hg) was similar with EPR oximetry and OxyLite. The muscle pO_2 did also increase during exercise, but to a lower extent, as the initial muscle pO_2 is already high (the pO_2 values measured using OxyLite probes were 19.8 ± 3.1 mm Hg and 26.8 ± 3.2 mm Hg for the control and stimulated group, respectively).

When mice were pretreated with an inhibitor of the NOS (L-NAME, 15 μmol.kg^{-1}, intraperitoneally) 1 h before the exercise protocol, the tumor pO_2 remained at the basal level, as demonstrated by EPR oximetry (Fig. 2).

Effect of the electrical stimulation protocol on the tumor blood flow

We used the OxyFlo technique, based on laser-doppler flowmetry, to assess the blood flow inside the tumor. The OxyFlo technique allows relative measurements of blood flow in arbitrary units (blood perfusion unit). We found that the tumor blood flow immediately increased after the beginning of the exercise protocol. Increases of 241 ± 18% and 162 ± 9% were observed for the TLT and FSaII tumor models, respectively. It decreased slowly during the 15 min of the protocol and immediately fell to the initial value just after the end of the stimulation (Fig. 3). The muscle blood flow showed an increase of 49 ± 13% during the sciatic nerve stimulation.

Effect of the electrical stimulation protocol on the tumor oxygen consumption rate

We measured the oxygen consumption rate of tumor cells extracted from FSaII tumors of mice 30 min after the end of the exercise protocol. We compared this rate with a control group. *In vivo* sciatic nerve stimulation reduced the rate of oxygen consumption by the tumor cells (Fig. 4). The mean slopes were −0.70 μM/min ± 0.01 and −0.30 ± 0.01 μM/min for control and electrical stimulated groups, respectively ($n = 4$ for each group). This means that *in vivo* stimulated tumor cells consumed oxygen 2.3 times slower than control tumor cells. We concluded that the tumor oxygenation increase was due to the decrease of the oxygen consumption by the tumor cells.

Effect of the electrical stimulation protocol on the sensitivity of tumors to irradiation

To determine whether electrical stimulation of the host tissue had an effect on the tumor response to radiotherapy,

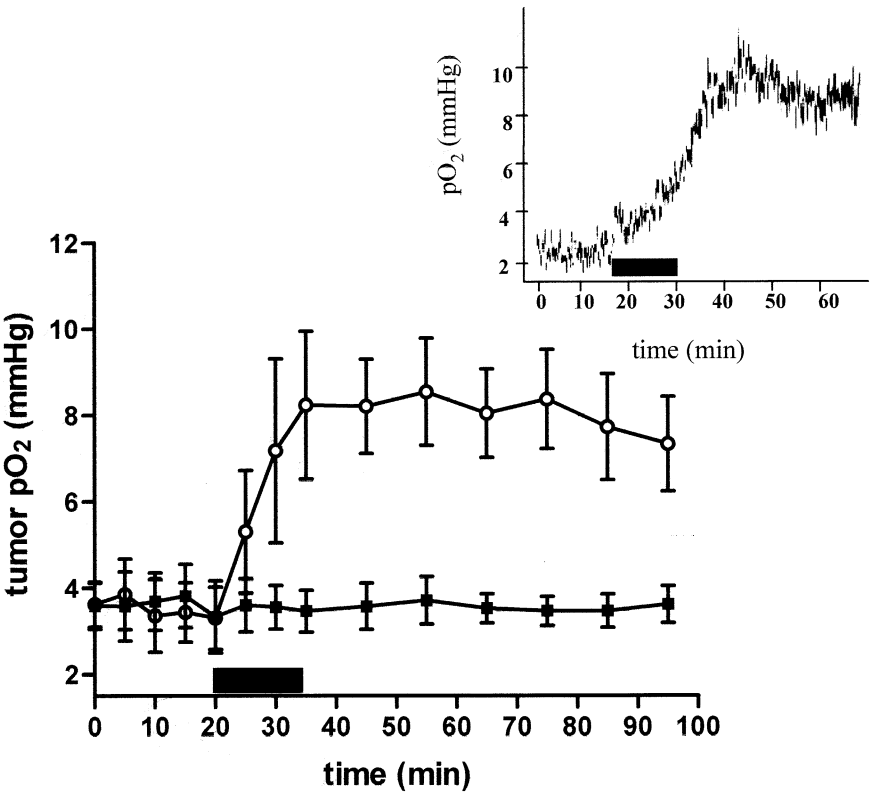

Fig. 1. Effect of the exercise protocol on the tumor pO_2: Graph: mean FSaII tumor pO_2 (±SEM) monitored by EPR oximetry ($n = 5$/group) before, during, and after electrical stimulation of the sciatic nerve. ○ stimulated group; ■ control group; black rectangle: stimulation period. Inset: typical FSaII tumor pO_2 monitored by OxyLite before, during, and after electrical stimulation of the sciatic nerve.

FSaII tumor-bearing mice were treated with irradiation alone, with the combination of electrical stimulation and irradiation, or with the combination of L-NAME 1 h before the stimulation protocol followed by irradiation, and the tumor regrowth delays were measured. As the FSaII tumor model is known to be radiosensitized by carbogen (29), we compared the effect of carbogen breathing during irradiation to the effects of electrical stimulation. To avoid tumor cure but still achieve a measurable regrowth delay, a single irradiation dose of 16 Gy of X-rays was selected as the radiation dose after preliminary tests. The regrowth delay to reach 12-mm tumor diameter was 3.9 ± 1.2 days for X-rays alone, 7.0 ± 0.8 days for carbogen and X-rays ($p < 0.05$), and 6.5 ± 0.6 days for electrical stimulation and irradiation ($p < 0.05$) (Fig. 5). These data indicate that electrical stimulation of the host tissue increased the sensitivity of tumors to X-ray irradiation, increasing regrowth delay by a factor of 1.65 compared with a factor of 1.79 for carbogen, treatment that is currently being used successfully in the clinic. The L-NAME pretreated group, further stimulated

Table 1. Effect of the exercise protocol on the tumor pO_2

		EPR		Oxylite	
Tumor model	Treatment	Pre (mm Hg)	Post (30 min) (mm Hg)	Pre (mm Hg)	Post (30 min) (mm Hg)
TLT	stimulation	3.3 ± 0.3	7.7 ± 0.5*	2.8 ± 1.2	8.1 ±2.2*
	L-NAME + stimulation	2.7 ± 0.2	3.3 ± 0.1	—	—
	control	1.1 ± 0.3	1.4 ± 0.3	1.9 ± 0.4	1.6 ± 0.5
FSaII	stimulation	3.1 ± 0.4	8.0 ± 0.4*	3.3 ± 0.3	7.3 ± 1.4*
	L-NAME + stimulation	2.0 ± 0.2	2.6 ± 0.2	—	—
	control	3.6 ± 0.6	3.5 ± 0.4	1.8 ± 0.7	1.9 ± 0.7

* $p < 0.05$ (Student t test)

Local tumor oxygenation measurements were carried out using two independent techniques: electron paramagnetic resonance (EPR) oximetry and a fiber-optic device (OxyLite). A 15-min exercise protocol was achieved by electrical stimulation at 5 Hz, with 0.2 ms duration pulses. L-NAME was injected intraperitoneally at 15 μmol.kg^{-1}, 1 h before the exercise protocol.

Abbreviations: EPR = electron paramagnetic resonance; TLT = local transplantable liver tumor; FSaII = fibrosarcoma.

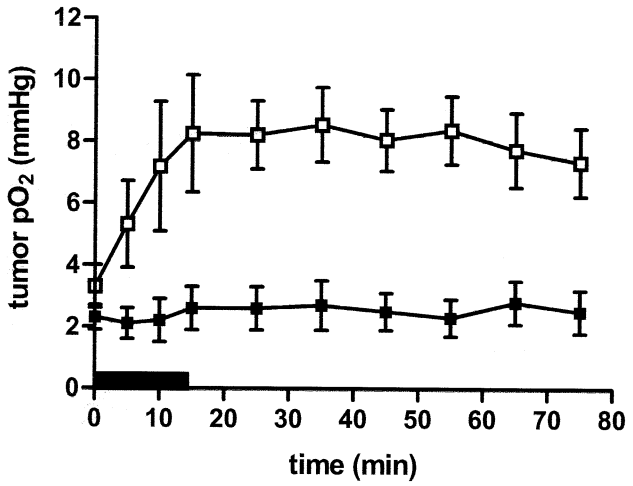

Fig. 2. Effect of the administration of an NOS inhibitor, L-NAME, 1 h before the exercise protocol on the FSaII tumor pO_2, measured by EPR oximetry. The results represent the mean pO_2 ± SEM measured during and after the stimulation period. □ electrical stimulation group; ■ L-NAME + electrical stimulation group.

electrically during irradiation, showed a regrowth delay of 5.3 ± 0.6 days (regrowth delay factor = 1.34). L-NAME administration before electrical stimulation partially inhibited the radiosensitizing effect of the electrical stimulation of the host tissue. L-NAME administration before irradiation (without any other treatment) had no effect on the tumor regrowth delay on this tumor model (30).

Effect of the electrical stimulation protocol on the tumor cGMP content and NO production

To compare the relative levels of NO produced by the tumor during this protocol, we performed *ex vivo* EPR spin-trapping after intravenous injection of the spin-trap agent MGD. The complex NO-Fe^{2+}(MGD)$_2$ has a characteristic triplet signal. The signal/noise ratio (maximum signal intensity/mean noise) was calculated for both groups

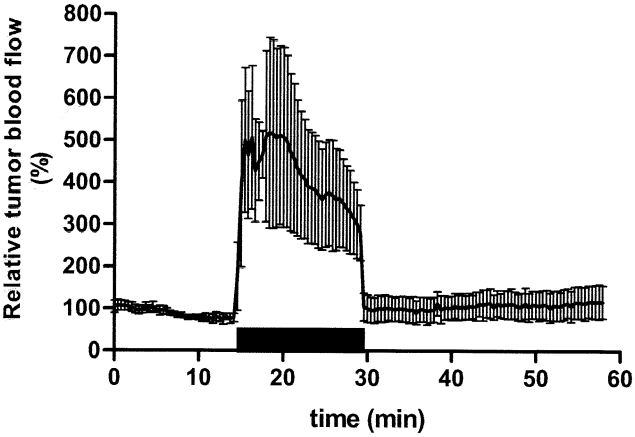

Fig. 3. Effect of the exercise protocol on the tumor blood flow: mean FSaII tumor blood flow (±SEM) measured by OxyFlo (n = 5/group) before, during, and after electrical stimulation of the sciatic nerve.

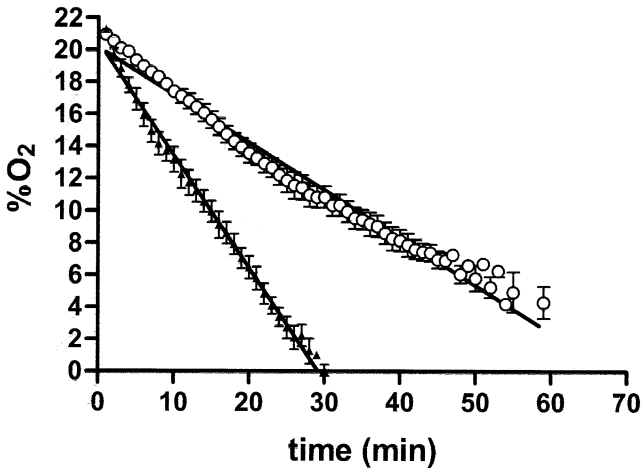

Fig. 4. Effect of the exercise protocol on tumor cell oxygen consumption rate. FSaII tumor cells' oxygen consumption rate (mean ± SEM, n = 4/group): ○ stimulated group, ▲ control group. Mean slopes: −0.70 μM/min ± 0.01 and −0.30 ± 0.01 μM/min for control and stimulated groups, respectively (n = 4/group). *In vivo* stimulated tumor cells consumed oxygen 2.3 times slower than control tumor cells.

(control and electrical stimulation). This ratio was divided by the tumor protein content. The value obtained for the control group was considered as a 100% value. We demonstrated that the electrical stimulation of the host tissue increased the relative tumor NO content (39.2% increase, $p < 0.05$). Moreover, we showed a concomitant increase in the tumor cGMP concentration during the electrical stimulation of the host tissue. Those results are presented in Fig. 6.

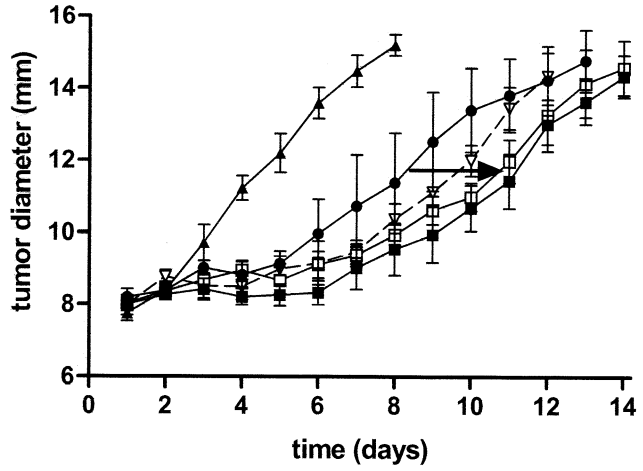

Fig. 5. Effect of the combination of the stimulation protocol and irradiation on FSaII tumor regrowth. Mice were untreated (▲), treated with 16 Gy of X-rays alone (●), electrically stimulated during irradiation with 16 Gy of X-rays (□), treated with carbogen 15 min before and during irradiation with 16 Gy of X-rays (■), or pretreated with L-NAME 1 h before the stimulation protocol and irradiated with 16 Gy of X-rays (▽). Each point represents the mean tumor size ± SEM of 5 tumors.

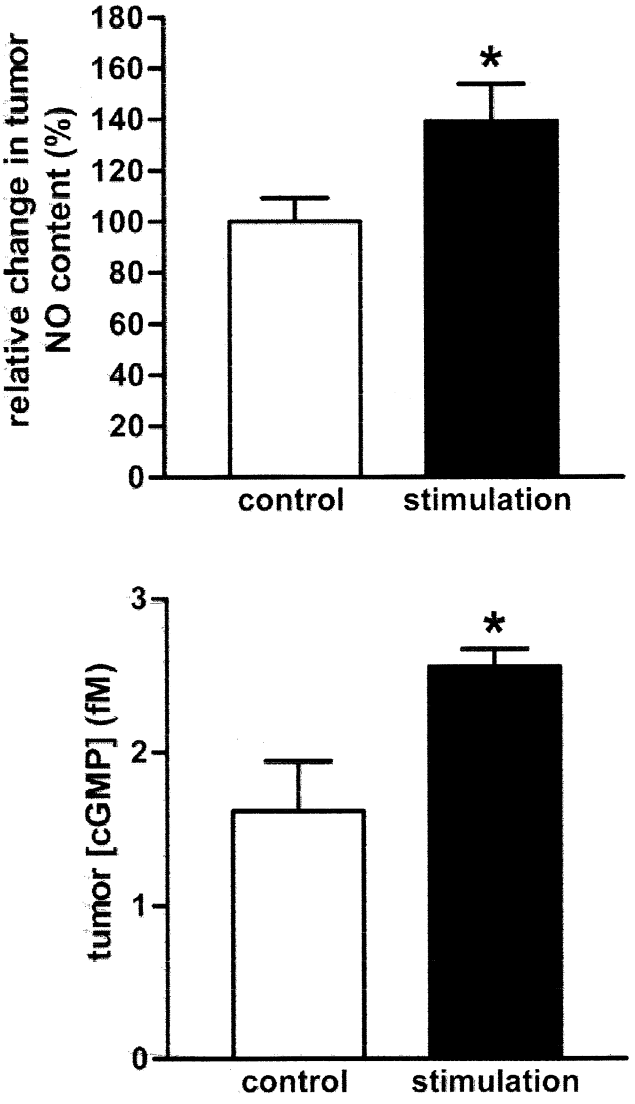

Fig. 6. Upper panel: Effect of the stimulation protocol on the relative change in tumor NO content. This was performed by *ex vivo* EPR spin-trapping after intravenous injection of the spin-trap agent MGD. The value obtained for the control group was considered as a 100% value. Lower panel: Effect of the stimulation protocol on the tumor cGMP concentration.

DISCUSSION

We report here that electrical stimulation of the host tissue does increase tumor blood flow and oxygenation during the exercise protocol, and that the increase in pO_2 is maintained at least 30 min after the sciatic nerve stimulation. The sudden increase in tumor blood flow that occurs when we initiate the stimulation protocol can explain the fast increase in tumor pO_2. As the increase in tumor blood flow is abolished immediately after the switch-off of the electrical stimulation, we cannot explain a prolonged effect on the tumor oxygenation only by a flow effect. The increase in tumor oxygenation is also related to the decrease in oxygen consumption by the tumor cells. It has indeed been predicted theoretically that modification of oxygen consumption is much more efficient at affecting oxygen transport than modification of delivery (31). We further demonstrated that a pretreatment with L-NAME, a NOS inhibitor, abolished almost totally the effect of exercise on the tumor oxygenation. The increase in the relative tumor NO content during the stimulation protocol, as demonstrated using EPR spin-trapping, and the increase in the tumor cGMP concentration both confirmed the NO-mediated pathway of this effect. We can conclude that the mechanism of the inhibition of oxygen consumption is likely to assign to the stimulation of the NOS during exercise. It has indeed been found that low concentrations of NO inhibit cytochrome oxidase (complex IV in the mitochondrial respiratory chain) reversibly and competitively with molecular oxygen (32). NO that is endogenously generated has been shown to inhibit respiration, and inhibitors of NOS have been shown to enhance respiration (33, 34). So, the NO/O_2 ratio at the level of cytochrome oxidase can provide a sensing mechanism for O_2 that will regulate oxygen consumption at the precise point at which it is used. Low concentrations of NO, continuously generated by NOS, explains the decrease in respiration that occurs at low oxygen concentration (34). Moreover, exposure of cells to NO can lead to inhibition of complex I concomitantly to complex IV. This seems to be due to S-nitrosylation of this enzyme and becomes progressively persistent as the concentration of reduced glutathione in the cell decreases. In this case, the likely scenario is that the stimulation protocol induced a production of NO and could thus decrease cell respiration (the basal pO_2 of the tumor being very low). We demonstrated a decrease in the tumor cells oxygen consumption rate after the protocol. This decrease in oxygen consumption could hence allow a maintaining of the increase in tumor pO_2 as observed in our studies.

One straightforward application of the present study is to take advantage of the increase in tumor blood flow during electrical stimulation. This could allow a better access of chemosensitizing agents to the tumor. Electrochemotherapy, i.e., intratumoral administration of a chemosensitizing agent followed by delivery of high-voltage electric pulses to the tumor, has the obvious aim to enhance the delivery of the drug into tumor cells owing to the permeabilization of the cell membrane. Besides that, Sersa *et al.* (35) studied the effect of these electric pulses on tumor blood flow. Although they observed a blood flow reduction at the amplitude of electric pulses usually applied for electrochemotherapy (antivascular effect), they further demonstrated that a modification of the amplitude resulted in changes in the direction of tumor blood flow response. It increased following pulses in the range between 80 and 560 V and decreased at higher amplitudes. This demonstrates that the local application of electric pulses to solid tumors can modify tumor blood flow and can be exploited either for an enhanced delivery of drugs or to increase tumor cytotoxicity. The stimulation protocol we used was carried out in the host tissue (muscle). Because this clearly increased tumor blood flow in the two tumor models under investiga-

tion, it should be considered for an enhancement in drug delivery to the tumor.

A second application of this study is to exploit the benefit in terms of radiosensitivity because the tumor regrowth delays increased during the stimulation protocol. We indeed demonstrated interesting radiosensitizing properties of this protocol. The increase in tumor oxygenation was demonstrated to be related to a decrease in tumor cells' respiration rate. Drugs that inhibit respiration, such as meta-iodobenzylguanidine (MIBG), were proposed as potential radiosensitizing agents (36). Mild hyperglycemia, which has also been demonstrated to reduce oxygen consumption (Crabtree effect), has been tested in combination with hyperoxic gas (37). Finally, we recently demonstrated that insulin infusion also had an inhibitory effect on the tumor cells' oxygen consumption rate and that this decrease in respiration resulted in an increase of the tumor oxygenation but also of the radiation response of the tumor. We demonstrated that the mechanism underlying this inhibition of tumor oxygen consumption rate was also NO mediated (30).

Because electrochemotherapy is already used in clinical practice (38), the use of such a protocol could be rapidly tested in patients. The main differences between electrochemotherapy and our electrical stimulation protocol rest in a lower voltage and a longer stimulation time.

REFERENCES

1. Vaupel P, Kallinowski F, Okunieff P. Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: A review. *Cancer Res* 1989;49:6449–6465.
2. Kimura H, Braun RD, Ong ET, *et al.* Fluctuations in red cell flux in tumor microvessels can lead to transient hypoxia and reoxygenation in tumor parenchyma. *Cancer Res* 1996;56: 5522–5528.
3. Dewhirst MW. Concepts of oxygen transport at the microcirculatory level. *Sem Rad Oncol* 1998;8:143–150.
4. Trotter MJ, Chaplin DJ, Durand RE, *et al.* The use of fluorescent probes to identify regions of transient perfusion in murine tumors. *Int J Radiat Oncol Biol Phys* 1989;16:931–935.
5. Hill SA, Pigott KH, Saunders MI, *et al*. Microregional blood flow fluctuations in murine and human tumours assessed using laser-doppler microprobes. *Br J Cancer* 1996; 27(Suppl. 74): 260–263.
6. Chaplin DJ, Hill SA, Bell KM, *et al.* Modification of tumor blood flow: Current status and future directions. *Semin Radiat Oncol* 1998;8:151–163.
7. Gallez B, Jordan BF, Baudelet C, *et al.* Pharmacological modifications of the partial pressure of oxygen in murine tumors: Evaluation using in vivo EPR oximetry. *Magn Reson Med* 1999;42:627–630.
8. Wijffels KI, Kaanders JH, Rijken PF, *et al.* Vascular architecture and hypoxic profiles in human head and neck squamous cell carcinomas. *Br J Cancer* 2000;83:674–683.
9. van der Sanden BP, Heerschap A, Hoofd L, *et al.* Effect of carbogen breathing on the physiological profile of human glioma xenografts. *Magn Reson Med* 1999;42:490–499.
10. Brown JM. The hypoxic cell: A target for selective cancer therapy—eighteenth Bruce F Cain Memorial Award lecture. *Cancer Res* 1999;59:5863–5870.
11. Koller A, Huang A, Sun D, *et al.* Exercise training augments flow-dependent dilation in rat skeletal muscle arterioles: Role of endothelial nitric oxide and prostaglandins. *Circ Res* 1995; 76:544–550.
12. Miller BF, Gruben KG, Morgan BJ. Circulatory responses to voluntary and electrically induced muscle contractions in humans. *Phys Ther* 2000;80:53–60.
13. Ament W, Lubbers J, Rakhorst G, *et al.* Skeletal muscle perfusion measured by positron emission tomography during exercise. *Eur J Physiol* 1998;436:653–658.
14. Kingwell BA. Nitric oxide-mediated metabolic regulation during exercise: Effects of training in health and cardiovascular disease. *FASEB J* 2000;14:1685–1696.
15. Gilligan DM, Panza JA, Kilcoyne CM, *et al.* Contribution of endothelium-derived nitric oxide to exercise-induced vasodilation. *Circulation* 1994;90:2853–2858.
16. Roberts CK, Barnard RJ, Jasman A, *et al.* Acute exercise increases nitric oxide synthase activity in skeletal muscle. *Am J Physiol* 1999;277:E390–394.
17. Swartz HM, Clarkson RB. The measurement of oxygen in vivo using EPR techniques. *Phys Med Biol* 1998;43:1957–1975.
18. Collingridge DR, Young WK, Vojnovic B, *et al.* Measurement of tumor oxygenation: A comparison between polarographic needle electrodes and a time-resolved luminescence-based optical sensor. *Radiat Res* 1997;147:329–334.
19. Griffiths JR, Robinson SP. The OxyLite: A fibre-optic oxygen sensor. *Br J Radiol* 1999;72:627–630.
20. James PE, Jackson SK, Grinberg OY, *et al.* The effects of endotoxin on oxygen consumption of various cell types in vitro: An EPR oximetry study. *Free Rad Biol Med* 1995;18: 641–647.
21. Taper H, Woolley GW, Teller MN, *et al.* A new transplantable mouse liver tumor of spontaneous origin. *Cancer Res* 1966; 26:143–148.
22. Volpe JP, Hunter N, Basic I, *et al.* Metastatic properties of murine sarcomas and carcinomas I: Positive correlation with lung colonization and lack of correlation with s.c. tumor take. *Clin Expl Metastasis* 1985;3:281–294.
23. Goudemant JF, Deconinck N, Tinsley JM, *et al.* Expression of truncated utrophin improves pH recovery in exercising muscles of dystrophic mdx mice: A 31P NMR study. *Neuromuscul Disord* 1998;8:371–379.
24. Conner EM. Duration of the hemodynamic effects of N(G)-nitro-L-arginine methyl ester in vivo. *Nitric Oxide Biol Chem* 2000;4:85–93.
25. Jordan BF, Baudelet C, Gallez B. Carbon-centered radicals as oxygen sensors for in vivo electron paramagnetic resonance: Screening for an optimal probe among commercially available charcoals. *Magn Reson Mater Phys Med Biol* 1998;7:121–129.
26. Jordan BF, Misson P, Demeure R, *et al.* Changes in tumor oxygenation/perfusion induced by the NO donor, isosorbide dinitrate, in comparison with carbogen: Monitoring by EPR and MRI. *Int J Radiat Oncol Biol Phys* 2000;48:565–570.
27. Tsuchiya K, Jiang JJ, Yoshizumi M, *et al.* Nitric oxide forming reactions of the water soluble nitric oxide spin-trapping agent, MGD. *Free Rad Biol Med* 1999;27:347–355.
28. Fujii H, Koscielniak J, Berliner LJ. Determination and characterization of nitric oxide generation in mice by in vivo L-Band EPR spectroscopy. *Magn Reson Med* 1997;38:565–568.
29. Griffin RJ, Okajima K, Song CW. The optimal combination of hyperthermia and carbogen breathing to increase tumor oxy-

genation and radiation response. *Int J Radiat Oncol Biol Phys* 1998;42:865–869.
30. Jordan BF, Grégoire V, Demeure R, *et al.* Insulin increases the sensitivity of tumors to irradiation: Involvement of an increase in tumor oxygenation mediated by a NO dependent decrease of the tumors cells oxygen consumption. *Cancer Res* 2002; 62:3555–3561.
31. Secomb TW, Hsu R, Ong ET, *et al.* Analysis of the effects of oxygen supply and demand on hypoxic fraction in tumors. *Acta Oncol* 1995;34:313–316.
32. Moncada S, Erusalimsky JD. Does nitric oxide modulate mitochondrial energy generation and apoptosis? *Nature Rev* 2002;3:214–220.
33. Shen W, Hintze TH, Wolin MS. Nitric oxide, an important signalling mechanism between vascular endothelium and parenchymal cells in the regulation of oxygen consumption. *Circulation* 1995;92:3505–3512.
34. Clementi E, Brown GC, Foxwell N, *et al.* On the mechanism by which vascular endothelial cells regulate their oxygen consumption. *Proc Natl Acad Sci USA* 1999;96:1559–1562.
35. Sersa G, Cemazar M, Parkins CS, *et al.* Tumour blood flow changes induced by application of electric pulses. *Eur J Cancer* 1999;35:672–677.
36. Biaglow JE, Manevich Y, Leeper D, *et al.* MIBG inhibits respiration: Potential for radio- and hyperthermic sensitization. *Int J Radiat Oncol Biol Phys* 1998;42:871–876.
37. Snyder SA, Lanzen JL, Braun RD, *et al.* Simultaneous administration of glucose and hyperoxic gas achieves greater improvement in tumor oxygenation than hyperoxic gas alone. *Int J Radiat Oncol Biol Phys* 2001;51:494–506.
38. Rodriguez-Cuevas S, Barroso-Bravo S, Almanza-Estrada J, *et al.* Electrochemotherapy in primary and metastatic skin tumors: Phase II trial using intralesional bleomycin. *Arch Med Res* 2001;32:273–276.