Glucocorticoids Modulate Tumor Radiation Response through a Decrease in Tumor Oxygen Consumption

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Abstract

Purpose: We hypothesized that glucocorticoids may enhance tumor radiosensitivity by increasing tumor oxygenation (pO_2) through inhibition of mitochondrial respiration.

Experimental Design: The effect of three glucocorticoids (hydrocortisone, dexamethasone, and prednisolone) on pO_2 was studied in murine TLT liver tumors and FSaII fibrosarcomas. At the time of maximum pO_2 (t_{max}, 30 min after administration), perfusion, oxygen consumption, and radiation sensitivity were studied. Local pO_2 measurements were done using electron paramagnetic resonance. The oxygen consumption rate of tumor cells after *in vivo* glucocorticoid administration was measured using high-frequency electron paramagnetic resonance. Tumor perfusion and permeability measurements were assessed by dynamic contrast-enhanced magnetic resonance imaging.

Results: All glucocorticoids tested caused a rapid increase in pO_2. At t_{max}, tumor perfusion decreased, indicating that the increase in pO_2 was not caused by an increase in oxygen supply. Also at t_{max}, global oxygen consumption decreased. When irradiation (25 Gy) was applied at t_{max}, the tumor radiosensitivity was enhanced (regrowth delay increased by a factor of 1.7).

Conclusion: These results show the potential usefulness of the administration of glucocorticoids before irradiation.

Tumor hypoxia is a critical determinant of resistance to radiotherapy and chemotherapy (1, 2). To target this resistance, prodrugs have been developed that are activated in hypoxic regions (3). In addition to this approach, we may also consider that a *transient* increase in tumor oxygenation may be beneficial if combined with radiotherapy. Indeed, a number of tumor oxygenating treatments have been developed to improve the therapeutic outcome. Mechanistically, tumor hypoxia results from an imbalance between oxygen delivery and oxygen consumption, either of which may be potentially targeted by therapeutic interventions. On one hand, oxygen delivery may be increased by increasing tumor perfusion (4–7) or by changing the hemoglobin saturation curve (8, 9). On the other hand, tumor hypoxia can be alleviated by decreasing the oxygen consumption. It has been predicted that modification of oxygen consumption is much more efficient at alleviating hypoxia than modification of oxygen delivery (10). Several drugs that inhibit mitochondrial respiration, such as metaiodobenzylguanidine (11), insulin (12, 13), and cyclooxygenase-2 inhibitors (14), have been characterized for their potential to increase tumor oxygenation and thereby enhance radiosensitivity.

Here, we hypothesized that glucocorticoids could be other important modulators of tumor oxygenation. The rationale for this hypothesis is that glucocorticoids are known to inhibit oxidative phosphorylation of the respiratory chain, with important effect on respiration rate of cells (15, 16). Using two different tumor models, we show that the administration of glucocorticoids (hydrocortisone, prednisolone, and dexamethasone) has a profound effect on tumor oxygenation. To identify the factors responsible for this tumor reoxygenation, we characterized changes in the tumor microenvironment: perfusion, permeability, and oxygen consumption. We also investigated the sensitivity of tumors to irradiation at the time of maximal reoxygenation.

Materials and Methods

Animal tumor models

Two different syngeneic tumor models were implanted in the gastrocnemius muscle in the rear leg of male mice (20-25 g; B&K, Hull, United Kingdom): the transplantable liver tumor TLT in NMRI mice and the FSaII tumor in C3H mice. All treatments were applied

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when the tumor reached 8.0 ± 0.5 mm. All experiments were conducted according to national animal care regulations.

Treatments

Anesthesia. Animals were anesthetized by inhalation of isoflurane mixed with 21% oxygen in a continuous flow (1.5 L/h) delivered by a nose cone. Induction of anesthesia was done using 3% isoflurane and stabilized at 1.2% for a minimum of 15 min before any measurement. The temperature of the animals was kept constant using an IR light, a homeothermic blanket control unit, or a flow of temperature-controlled warm air.

Glucocorticoids. All glucocorticoids were administered by 100-μL i.p. injection. Hydrocortisone was administered at 7.7 mg/kg (Solu-Cortef, Pharmacia, Pfizer, Brussels, Belgium; diluted in saline to 1.9 mg/mL), dexamethasone at 5 mg/kg (Aacidexam, Organon, Brussels, Belgium; diluted in saline to 1.25 mg/mL), and prednisolone at 75 mg/kg (Alexis Biochemicals, Zandhoven, Belgium; diluted in saline to 25 mg/mL).

Oxygen measurements

Electron paramagnetic resonance (EPR) oximetry (using charcoal as the oxygen sensitive probe) was used to evaluate tumor oxygenation changes as previously described (6, 17). EPR spectra were recorded using a 1.2-GHz EPR spectrometer (Magnettech, Berlin, Germany) before and after administration of glucocorticoids. Two days before EPR analysis, mice were injected in the center of the tumor using the suspension of charcoal (100 mg/mL, 50 μL injected; particle size, <25 μm). These localized EPR measurements record the average pO_2 in a volume of ~10 mm^3 (6). Hydrocortisone was administered in FSaII tumor– and TLT tumor–bearing mice (n = 5 per group). Prednisolone and dexamethasone were administered in FSaII tumor–bearing mice (n = 4 per group). Saline was administered for both tumor types (n = 4 per group).

Oxygen consumption rate evaluation

The preparation of the tumor cells was as follows. FSaII tumors were dissected in sterile environment and gently pieced in McCoy's medium.

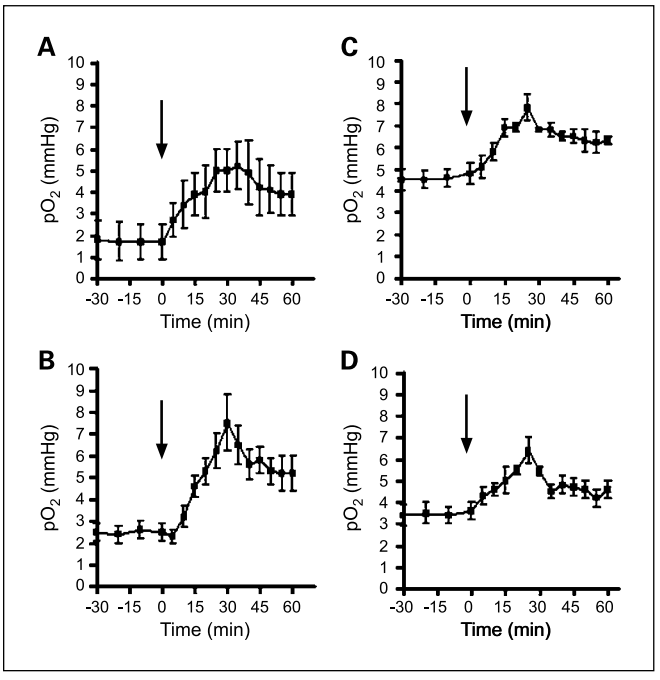

Fig. 1. Tumor pO_2 measured by EPR oximetry as a function of time in TLT tumors treated with hydrocortisone (n = 5; *A*); FSaII tumors treated with hydrocortisone (n = 5; *B*); FSaII tumors treated with dexamethasone (n = 4; *C*); and FSaII tumors treated with prednisolone (n = 4; *D*). Arrows, injection time of the drug.

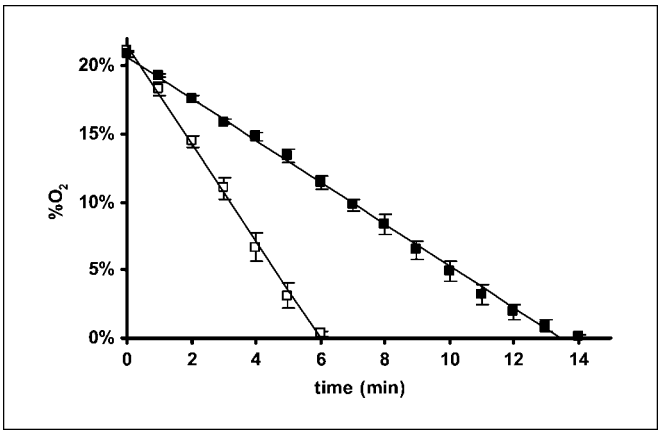

Fig. 2. Effect of hydrocortisone administration on tumor oxygen consumption rate in FSaII cells. Hydrocortisone-pretreated cells consumed oxygen significantly more slowly than cells treated with saline alone. □, saline (n = 5); ■, hydrocortisone (n = 5). $P < 0.01$, Wilcoxon rank-sum test.

The cell suspension was filtered (100 μm pore size nylon filter; Millipore, Brussels, Belgium), centrifuged (5 min, 450 × g, 4°C), and cells were set in culture in DMEM containing 10% fetal bovine serum. Confluent cells were suspended in medium without serum 2 h before treatment with hydrocortisone (5.2 μmol/L) or saline. Thirty minutes after treatment, they were trypsinized and cell viability was determined by trypan blue exclusion. An EPR method was used, which has previously been described (12). Briefly, the spectra were recorded on a Bruker EMX EPR spectrometer operating at 9 GHz. Cells (2×10^7/mL) were suspended in 10% dextran in complete medium. A neutrite nitroxide, ^{15}N 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-15N-1-oxyl at 0.2 mmol/L (CDN Isotopes, Pointe-Claire, Quebec, Canada), was added to 100-μL aliquots of tumor cells that were then drawn into glass capillary tubes. The probe was previously calibrated so that the line width measurements could be related to O_2 (12). The sealed tubes were placed into quartz EPR tubes and the samples were maintained at 37°C. Because the resulting line width reports on pO_2, it was possible to calculate oxygen consumption rates by measuring the pO_2 in the closed tube as a function of time and subsequently compute the slope of the resulting plot.

Perfusion measurements

Patent Blue staining. Patent Blue (Sigma-Aldrich, Bornem, Belgium) was used to obtain a rough estimate of the FSaII tumor perfusion (18) 30 min after administration of hydrocortisone (n = 7) or saline (n = 5). This technique involves the injection of 200 μL of Patent Blue solution (1.25%) into the tail vein of the mice. After 1 min, a uniform distribution of the staining throughout the body was obtained and mice were sacrificed. Tumors were carefully excised and cut in two size-matched halves. Pictures of each tumor cross section were taken with a digital camera. To compare the stained versus unstained area, an in-house program running on IDL (Interactive Data Language, RSI, Boulder, CO) was developed. For each tumor, a region of interest (stained area) was defined on the two pictures and the percentage of stained area of the whole cross section was determined (19). The mean of the percentage of the two pictures was then calculated and used as an indicator of tumor perfusion.

Magnetic resonance imaging measurements. The FSaII tumor perfusion was monitored 30 min after injection of hydrocortisone (n = 6) or saline (n = 6) via single-slice dynamic contrast-enhanced magnetic resonance imaging (MRI) at 4.7 T using the rapid-clearance blood pool agent P792 (Vistarem, Guerbet, Roissy, France; ref. 20). High-resolution multislice T_2-weighted spin-echo anatomic imaging was done just before dynamic contrast-enhanced T_1-weighted gradient-recalled echo imaging. T_1-weighted gradient-recalled echo images were obtained

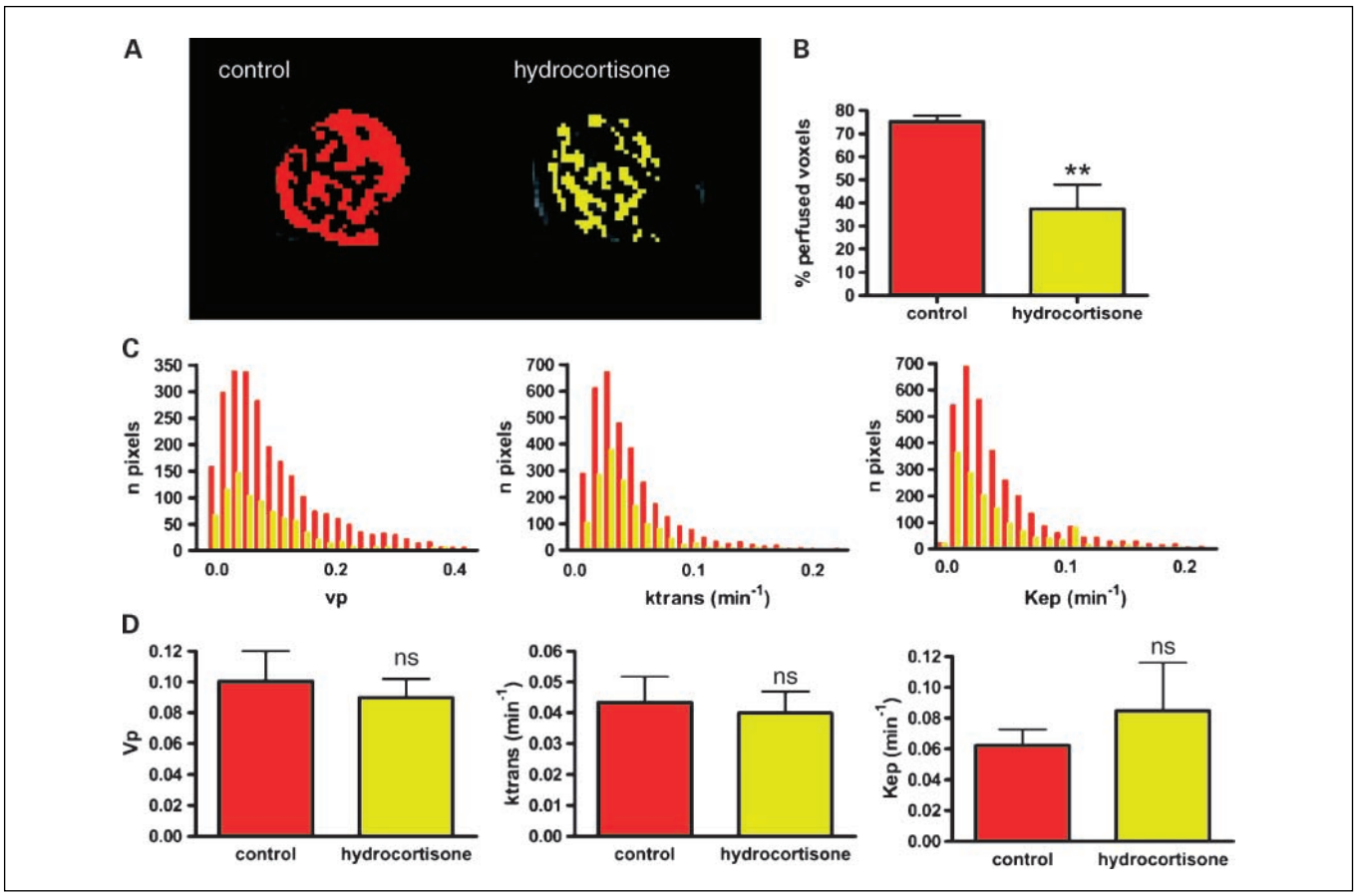

Fig. 3. Perfusion and permeability were assessed in FSaII tumors by dynamic contrast-enhanced MRI with P792 as the contrast agent in FSaII tumor – bearing mice 30 min after saline ($n = 6$) or hydrocortisone ($n = 6$) treatment. *A*, typical MRI images of FSaII tumors showing the perfused pixels 30 min after treatment. *B*, columns, mean percentage of perfused pixels for treated and control groups; bars, SE. **, $P < 0.01$, Wilcoxon rank-sum test. *C*, distribution of pharmacokinetics variables in tumors treated with saline or hydrocortisone. *D*, columns, overall estimation of pharmacokinetic variables in tumors; bars, SE. Statistical analysis done with Wilcoxon rank-sum test; NS, nonsignificant.

using the following variables: repetition time, 40 ms; echo time, 4.9 ms; slice thickness, 1.6 mm; flip angle, 90 degrees; matrix, 64 × 64; field of view, 4 cm; acquisition time, 2.56 s per scan. Pixel-by-pixel values for K_{trans} (influx volume transfer constant, from plasma into the interstitial space, units of min^{-1}), V_p (blood plasma volume per unit volume of tissue, unitless), and k_{ep} (fractional rate of efflux from the interstitial space back to blood, units of min^{-1}) in tumor were calculated via tracer kinetic modeling of the dynamic contrast-enhanced data, and the resulting parametric maps for K_{trans}, V_p, and k_{ep} were generated. Statistical significance for V_p or K_{trans} identified "perfused" pixels (i.e., pixels to which the contrast agent P792 had access; refs. 20, 21).

Tumor regrowth delay assay. The FSaII was locally irradiated with a 250-kV X-ray irradiator (RT 250, Philips Medical system; 1.2 Gy/min, 25 Gy). The tumor was centered in a 3-cm-diameter circular irradiation field. After treatment, tumor diameter was measured every day using a digital caliper until the diameter reached 16 mm, at which time the mice were sacrificed. A linear fit was done for diameters ranging from 8 to 16 mm, allowing determination of the time to reach a particular size for each mouse. Four groups of FSaII tumor – bearing mice were used for this study: saline ($n = 7$), hydrocortisone ($n = 7$), saline 30 min before irradiation ($n = 6$), and hydrocortisone 30 min before irradiation ($n = 5$).

Clonogenic cell survival assay

FSaII tumors were dissected in sterile environment and gently pieced in McCoy's medium. The cell suspension was filtered (100 μm pore size nylon filter; Millipore), centrifuged (5 min, 450 × *g*, 4°C), and cells were set in culture in DMEM containing 10% fetal bovine serum. Confluent cells were treated with hydrocortisone (5.24 μmol/L) or saline 30 min before irradiation (2, 5, 8, or 12 Gy). The cells were washed and reincubated in the conditioned medium without drug 1 h after irradiation. After 7 days of incubation in a humidified 5% CO_2 atmosphere at 37°C, the dishes were stained with crystal violet. All colonies formed by >50 cells were counted.

Statistical analysis

The change in pO_2 was assessed by Wilcoxon signed-rank test. The oxygen consumption slopes were compared by a Wilcoxon rank-sum test. The dynamic contrast-enhanced MRI variables and the Patent Blue results were compared by Wilcoxon rank-sum test. For the regrowth delay study, a one-way ANOVA Tukey's multiple comparison test was applied, and for clonogenic cell survival assay, a two-way ANOVA test was applied.

Results

Effect of hydrocortisone on the tumor oxygenation. Hydrocortisone, dexamethasone, and prednisolone were tested for their possible effect on tumor oxygenation. After administration of hydrocortisone, we observed a rapid increase in pO_2 in both TLT and FSaII tumor models (Fig. 1A and B) that was not observed for saline groups. The same kind of kinetics was observed after administration of dexamethasone and

prednisolone in FSaII tumors (Fig. 1C and D). The maximal pO_2 was reached ~30 min after treatment. This value was significantly higher than that before treatment ($P < 0.05$) for all the drugs tested. The tumor pO_2 remained elevated until at least 1 h after administration.

Effect of hydrocortisone on oxygen consumption. To determine if the increase in pO_2 is due to a decrease in tumor oxygen consumption by the tumor cells, the tumor oxygen consumption was investigated 30 min after treatment. The administration of hydrocortisone significantly decreased the rate of oxygen consumption (Fig. 2): 3.59 ± 0.17 μmol/L/min for the control group versus 1.48 ± 0.03 μmol/L/min for the treated group (mean ± SE; $P < 0.01$, $n = 5$).

Effect of hydrocortisone on hemodynamic variables. The blood perfusion and vascular permeability of tumor was investigated using dynamic contrast-enhanced MRI 30 min after hydrocortisone administration. A significant decrease of 37.7 ± 10.8% ($P < 0.01$) in the percentage of perfused pixels was observed 30 min after injection, showing that perfusion is significantly reduced by hydrocortisone (Fig. 3A and B). The values of plasmatic volume fraction (V_p) and permeability (K_{trans} and k_{ep}) were unchanged (Fig. 3C and D). These results were further confirmed by the Patent Blue assay. Tumors treated with hydrocortisone stained less positive (28.4 ± 5.1%) than control tumors (69.7 ± 6.1%). This difference was found to be statistically significant ($P < 0.01$).

Improvement of radiation efficacy. To determine whether hydrocortisone had an effect on the tumor response to radiotherapy, FSaII tumor–bearing mice were treated with saline alone, hydrocortisone alone, with irradiation plus saline, or with irradiation plus hydrocortisone. The regrowth delay assay is shown in Fig. 4. There was no significant difference between tumors treated with saline or hydrocortisone ($P > 0.05$). All irradiated groups showed a significant ($P < 0.001$) regrowth delay in comparison with their control group. When combining irradiation with the administration of hydrocortisone at the time of maximal reoxygenation, the regrowth delay (8.46 ± 0.53 days) was significantly increased (by a factor of 1.7) compared with irradiation alone (4.84 ± 0.95 days; $P < 0.001$).

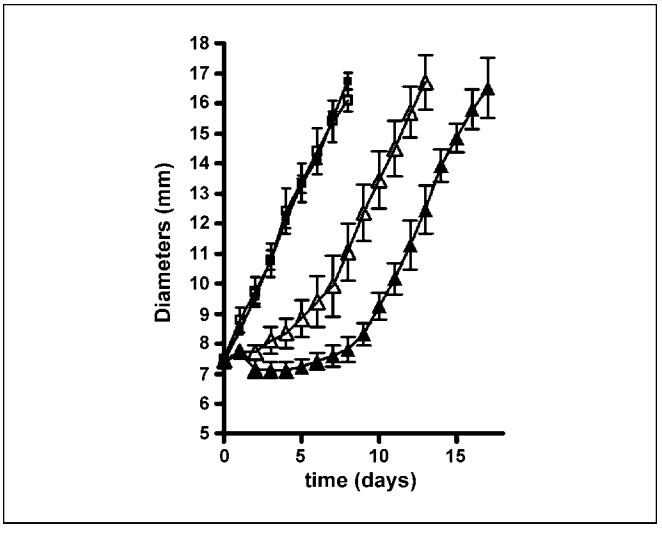

Fig. 4. Effect of hydrocortisone on the regrowth delay of four groups of FSaII tumors: control group only injected once with saline (□, $n = 7$); second group injected once with hydrocortisone (■, $n = 7$); third group treated with saline 30 min before 25 Gy irradiation (△, $n = 6$); and fourth group treated with hydrocortisone 30 min before 25 Gy irradiation (▲, $n = 5$).

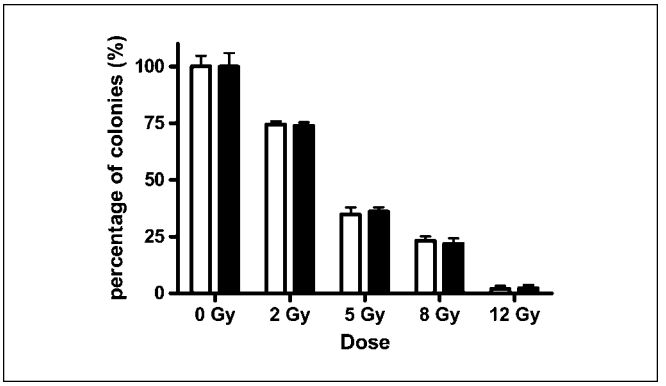

Fig. 5. A clonogenic cell survival assay was done on FSaII cells to discriminate between an oxygen effect and a direct radiosensitization effect. Compared with control cells, hydrocortisone did not exert any sensitizing effect. For all doses, irradiation led to a significant decrease ($P < 0.001$, two-way ANOVA). These observations show that hydrocortisone radiosensitizes tumors through changes in the tumor microenvironment rather than by a direct sensitizing effect. Columns, mean; bars, SE.

***Effect of hydrocortisone on FSaII radiosensitivity* in vitro.** To discriminate between an oxygen effect and a direct radiosensitization effect, a clonogenic cell survival assay was done on FSaII cells. Compared with control cells, hydrocortisone did not exert any sensitizing effect. The irradiation led to a significant decrease in the number of colonies depending on the irradiation dose ($P < 0.001$). These observations show that hydrocortisone radiosensitizes tumors through changes in the tumor microenvironment rather than by a direct sensitizing effect (Fig. 5).

Discussion

The two major findings of this study are that (*a*) the use of glucocorticoids can significantly promote tumor oxygenation by decreasing the local oxygen consumption, and (*b*) glucocorticoids can significantly increase the effectiveness of tumor radiotherapy when irradiation is given at the time of maximal reoxygenation.

For the first time, we report that glucocorticoids induce a significant increase in tumor oxygenation in two different tumor models (Fig. 1). This phenomenon occurs rapidly, within 30 min after administration (t_{max}). At t_{max}, multiple modalities were applied to determine the mechanisms responsible for this effect. Because a change in oxygenation could result from an increase in oxygen blood supply or a change in consumption rate, we investigated both variables. Our results show that the reoxygenation of the tumors is linked to an effect on oxygen consumption. This was determined by measuring the oxygen consumption rate by tumor cells (Fig. 2): we found that oxygen consumption was significantly reduced after *in vivo* administration of hydrocortisone. This decrease in oxygen consumption could be explained by the capacity of glucocorticoids to inhibit cytochrome *c* oxidase (complex IV) of the mitochondrial respiratory chain (15, 16). At this point, we may also note the remarkable similarity of this effect between

nonsteroidal anti-inflammatory drugs (14) and glucocorticoids (the present study). Both classes of drugs inhibit the prostaglandin cascade at two different levels (inhibition of cyclooxygenase and inhibition of phospholipase A2). Although some studies have described a direct effect of these drugs with specific complexes of the mitochondrial respiratory chain, it is likely that unspecific effects may occur with most drugs that interfere with the prostaglandin cascade. Indeed, prostaglandins are known to play a major role in the mitochondrial respiration (22–25), and the inhibition of their production should lead to a similar decrease in oxygen consumption and tumor reoxygenation. Moreover, the results obtained from dynamic contrast-enhanced MRI (Fig. 3) and by the Patent Blue assay preclude the possibility that the increase in tumor oxygenation may also result from an increase in perfusion. This decrease in perfusion is not surprising because the anti-inflammatory effects of glucocorticoids are known to be associated with a reduction in the vascular tone (or with vasoconstriction). This phenomenon was already observed in the same tumor model using anti–cyclooxygenase-2 drugs (14). It is interesting to note that for both glucocorticoids and cyclooxygenase-2 inhibitors (14), the decrease in oxygen consumption was sufficient to counteract the decrease in perfusion, and that the balance was in favor of an increase in tumor oxygenation. Finally, we cannot exclude that glucocorticoids may also increase tumor oxygenation by additional mechanisms. For example, it is well known that chronic treatments using glucocorticoids could lead to hyperglycemia in some patients. The so-called "Crabtree effect" results in a reduction of oxygen consumption via respiration in favor of glycolysis. In chronic treatments using glucocorticoids, this effect could be additive to the effect on the tumor mitochondrial respiratory chain to explain an increase in tumor oxygenation. The effect of glucocorticoids on the response to irradiation has been a matter of debate in the literature. Some reports in the literature have suggested an increased radioresistance induced by some glucocorticoids (26–30). The mechanisms proposed for increased radioresistance include an effect on the cell cycle (26), a decrease in radio-induced apoptosis (27, 28), and metabolic changes (29). However, this radioprotective effect was observed in few cell lines (31, 32) and not in some others (32–34). Here, we did not find any direct radiosensitization effect *in vitro* when the tumor cells were incubated in the presence of glucocorticoids (Fig. 5). On the contrary, we observed an important radiosensitization *in vivo* (Fig. 4), indicating that this effect is clearly linked to an effect on the tumor microenvironment. To our knowledge, this radiosensitization effect induced by the glucocorticoids is described here for the first time. Moreover, the demonstration that the enhanced radiosensitivity is mediated by an oxygen effect predicts that the radiosensitization of normal tissue is unlikely. This radiosensitization will more than likely be higher for hypoxic tumor regions than for well-oxygenated tissues. Further preclinical studies should confirm these assumptions. For this purpose, several normal tissue models can be used to determine whether glucocorticoids are responsible for toxicity on early-responding tissues (e.g., intestinal regenerated crypt assay) or late-responding tissues (e.g., leg contracture assay).

Finally, the demonstration of an oxygen effect gives unique insights for treatment combinations in the clinic. Because glucocorticoids are commonly used during several cancer therapies (e.g., to treat edema associated with malignant glioma; refs. 35, 36), our study suggests a potential therapy benefit if those glucocorticoids were to be given just before the irradiation.

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