Role of Blood Flow vs. O_2 Consumption in Nicotinamide-induced Increase pO_2 in a Murine Tumor

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= Abstract =

We evaluated the effect of nicotinamide on cellular O_2 consumption and metabolic status i.e., adenylate phosphates and NAD^+ *in-vitro*, and changes in blood flow *in-vivo*, to determine whether changes in cellular metabolism or increased oxygen availability, was responsible for increased tumor oxygenation. Thirty min. pre-incubation of cells with~4mM (=500mg/kg) nicotinamide resulted in no change in cellular O_2 consumption. Similarly, neither the adenylate phosphates nor the cellular NAD^+ levels were altered in the presence of~4mM nicontinamide. *In-vivo*, nicotinamide (500mg/kg) increased O_2 availability as estimated by changes in relative tumor blood flow (RBC flux). The changes in RBC flux measured by the laser Doppler method, were tumor volume dependent and increased from~35% in~150mm^3 tumors to~75 % in~500mm^3 tumors. In conclusion, these observations indicate a reduction in local tissue O_2 consumption is not a mechanism of improved tumor oxygenation by nicotinamide in FSa II murine tumor model. The primary mechanism of increased pO_2 appears to be an increased local tumor blood flow.

Key Words : Nicotinamide, Oxygen consumption (Q_{O2}), Tumor blood flow (TBF), Laser Doppler flowmetry,

INTRODUCTION

Several investigators have shown that nicotinamide (the amide form of vitamin B_3) increase radiation sensitivity in various tumors[1-6]. This increased radiation sensitivity may be resulted from increased intratumor pO_2, as demonstrated using oxygen microelectrodes in several human tumor xenografts and murine tumors[1]. These studies indicate that the pO_2 increases as a result of increased tumor blood flow (TBF) and/or decrease in transient fluctuations in TBF[1-6].

However, in studies with the C3H mouse mammary carcinoma by Horsman et al.[7], nicotinamide did not increase TBF, but a drop in tumor energy status was observed. These authors suggested that the reduction in energy status may occur secondary to suppression of O_2 consumption (Q_{O2}) by nicotinamide, thus, leading

to an increased pO_2 in tumors. In view of these observations we evaluated the possibility that nicotinamide increases tumor oxygen status(in part) by decreasing tumor cell oxygen consumption[8-10]. In addition to the studies of Horsman et al.[7], the possibility of nicotinamide induced changes on O_2 consumption is indicated by its potential role as a precursor of nicotinamide adenine dinucleotide (NAD^+)[11-13], which serves as an electron carrier in oxidative phosphorylation. Additionally, NAD^+ is an essential coenzyme for several dehydrogenases, including lactate dehydrogenase. Alteration in the cellular level of NAD^+ may influence the relative rate of pyruvate metabolism to lactate. In the present study we examined the possibility that increased tumor pO_2 may result from an inhibition of O_2 consumption at the cellular level, and also examined the effect of nicotinamide on cellular energy status and cellular NAD^+ level. In addition to changes in O_2 consumption, we examined for possible nicotinamide induced changes in O_2 delivery by evaluating RBC flux in FSaII tumors as a function of tumor volume.

MATERIALS AND METHODS

Animals and Tumors Cells

Female C3Hf/Sed mice, 8-10 weeks of age, were used. Animals were maintained on a sterile standard laboratory diet and kept in under specific pathogen-free conditions. This study was conducted under Massachusetts General Hospital Animal Care Committee Regulations for animal welfare. Fourth generation FSaII tumors were transplanted s.c.into the right thigh of the mice[14]. The experiments were carried out when tumor volume was between~100 mm^3 and~800mm^3.

Measurements of Oxygen Consumption and Metabolic Status

FSaII tumor cells were cultured in plastic tissue culture flasks in Dulbecco modification of Eagle's medium at pH 7.4, and single cell suspensions were prepared using 0.05% Trypsin-EDTA. Oxygen uptake determinations were previously described[8]. Briefly, O_2 measurements were performed using a YSI Model 5300 Biological Oxygen monitor coupled to a YSI 5301 Standard Bath Assembly and YSI 5331 Standard Oxygen Probes (YSI Scientific, Yellow Springs, OH). For all experiments the water bath was kept at 37℃ and the probe was calibrated before and after use. Following trypsinization, cells were counted and a desired number of cells was sedimented for 4 min. at 4℃. The cells were resuspended and equilibrated with a 5% carbon dioxide/air mixture, treated with nicotinamide(~4 mM). The suspension was then placed in a warm room at 37℃ and agitated for 30min. A 3.5ml cell sample was placed in the bath-well and re-equilibrated with the air/carbon dioxide mixture. Caution was taken to remove the small air bubbles trapped between the well and probe. Readings on the amplifier monitor, expressed as percentage of initial O_2 saturation, were taken each min. for 6 to 10 consecutive min. In this study, all of our O_2 uptake determinations were in the range of 100 % O_2 saturation down to a low of 50% saturation[8,15]. Experiments, with or without nicotinamide, were conducted on the small cell suspension to ensure that small changes in O_2 uptake were not due to small differences in cell number. For the determination of cellular adenylate phosphates and NAD^+, the medium (±nicotin-amide for 30 min.) overlying cell monolayers was removed, the cells were washed, and immedia-tely frozen by submersion in liquid nitrogen. The surface of the frozen cells was overlain with 1.0 ml of 80% methyl alcohol in pH 8 tris buffer and warmed to —15℃. Cells were scraped and extracted for 10 min. and the supernatant was heat denatured for 15 sec in a boiling water bath. Following centrifugation the samples were analyzed by HPLC as previously described[8,15].

Anesthesia Procedure Prior to in vivo Experimental Protocol

The mice were completely anesthetized via i.m. route with a mixture of ketamine(90 mg/kg) and

xylazine(9 mg/kg) to avoid movement artifacts during the measurement(an injection volume: 0.2 ml/20g body weight). The temperature of the tumor was measured with a needle-type thermocouple, and the rectal temperature was measured with an implant-probe(encased in Teflon) thermocouple using a digital thermometer (Model BAT-10/Physitemp, Clifton, NJ, USA). After anesthetization, mice were placed on a heating pad to keep the body (rectal) temper-ature at~37.5℃. In sham controls both tumor and body temperatures were measured. Average tumor temperature was~34℃ when the body temperature was maintained at~37.5℃.

Measurement of Relative TBF (RBC flux)

Relative tumor blood flow (RBC flux) was measured using the laser Doppler needle probe (Model 433-1) and the Laserflow Blood Perfusion Monitor 403A (TSI, Inc., MN, USA). Briefly, a small hole was made using a 23 gauge needle and a 0.8 mm-diameter laser Doppler needle probe was inserted into the tumor's center. It was then slightly withdrawn to ensure that there was no compression of the tumor under the probe tip. The electrical signal of flow, velocity and volume from the laser Doppler systems were digitally processed using the Mac Lab/4 analog-digital system (ADInstruments, Castle Hill, New South Wales, Australia) linked to a Macintosh computer with output voltage ranging from 0 V to 2.5V. Relative TBF (RBC flux) was monitored for a period of 120 min following the injection of nicotinamide. In addition, the zero-flow signal was measured at the end of the experiments by sacrificing the animals with an overdose of anesthesia; the biological zero-flow signal was well-above the electrical zero (output signals were usually between~25mV and~150mV in dead mice).

Measurement of Mean Arterial Blood Pressure (MABP)

The right carotid artery was exposed and cannulated with a PE-10 polyethylene cathether (Clay Adams, Parsippany, NJ, USA), using a binocular microscope. During the insertion of PE-10 tubing into the artery, the artery was occluded with a small clamp. The tubing, previously filled with heparin (70 units/ml), was connected with a three-way stopcock to a pressure transducer (Gould, Inc., Valley View, OH, USA). The clamp on the artery was then released, and the PE-10 tubing was checked for blood and tied with a fine silk thread. The stopcock was then turned on to allow communication between the tubing and the pressure transducer. MABP was measured simultaneously with the measurement of RBC flux for at least 2 hr after the nicotinamide treatment[14]. To evaluate the impact of this level of anesthesia on macrohemodynamic parameters, the MABP and heart beat rate were measured in anesthetized and unanesthetized mice(in fact, recovered from the anesthesia, 4 hr after the anesthesia). An anesthesia with a mixture of ketamine and xylazine decreased the MABP from ~90 to ~75 mmHg, and also decreased the heart beat rate from 350 to 250 beat/min. For the measurement of RBC flux, mice should be anesthetized to avoid the artifacts from the animal movement. Furthermore, hypertensive stress reactions may influence the actual blood flow in the observation of conscious animals. Also, all of these artifacts in unanesthetized animals are random and beyond our control. From sham control (N=5), mice were anesthetized with an i.m. injection (an injection volume was 0.2 ml/20 g body weight) of a mixture of ketamine and xylazine, and placed on a heating pad to keep the body temperature~37.5℃. The preparation time for the arterial cannulation for the measurement of MABP took~10 min., and all mice were individu-ally connected with the pressure transducer. An additional dose of anesthesia (an injection volume: 0.1 ml) was given at every~60 min after the first injection. MABP was~75 mmHg after the anesthesia, and it slightly fluctuated without any significant reduction in MABP during 2 hr postinjection (Table 1).

Table 1. The Time Sequence for the Measurement of MABP and RBC Flux.

Time(min.) after the 1st anesthesia							
0	10	20	30	60	90	120	150
I.	II.	III.	*IV.	V.		VI.	VII.
Time(min.) after the nicotinamide (500mg/kg) treatment							
			*[0 min in Figures]		60		120

I. Injected with the 1st anesthesia (~0.2ml), and (~5 min later) began to cannulate the artery.
II. Finished the cannulation for the artery, and connected to the pressure transducer [MABP].
III. Inserted the laser Doppler needle [RBC flux].
IV. Injected with nicotinamide [0 min. in Figures]*.
V. Injected with the 2nd anesthesia (0.1 ml).
VI. Injected with the 3rd anesthesia (0.1 ml).
VII. Injected with 300mg/kg of sodium pentobarbital to sacrifice the mice.

Measurement of Hematocrit Levels (HCT)

The HCT was measured with a ~70μl blood sample (~3% of total blood volume), collected from the orbital sinus of each mouse[16]. The capillary tubes were centrifuged for 10 min at 12, 500rpm, and separate bands of HCT were read.

Data Analysis

All values are shown as mean±standard error (SE) of each group and time points for the parametric statistic. Relative (or percent changes were determined individually for each mouse, based on pre-treatment values, and then averaged. The significance of the differences within a group before and after the nicotinamide treatment was evaluated using a paired t-test. Significant differences between treatment groups were checked with an unpaired t-test. The level of significance was set at $p<.05$.

RESULTS

The mean O_2 consumption rate in the control FSaII tumor cells (N=5) was $2.18\pm0.12 \times 10^{-15}$ mole/cell/min. (Table 2). Preincubation of cells at 37℃ for 30 min. at a dosage of 500mg/kg of nicotinamide resulted in no change in O_2 consumption rate ($2.13\pm0.08\times10^{-15}$ mole/min/cell). Table 2 also shows the cellular concentration of ATP, ADP, AMP, adenylate energy charge (AEC) [ATP +0.5 ADP]/[ATP+ADP+ AMP], and cellular NAD^+ levels in the absence and presence of nicotinamide (30 min. prior to and during analysis).

Table 2. Metabolic Status in the Absence and Presence of Nicotinamide

	— Nicotinamide[a]	+ Nicotinamide[a]
Q_{O2}(10^{-15}moles/cell/min.)	2.18±0.12[b]	2.13±0.08
ATP(10^{-15}moles/cell)	4.46±0.28	4.53±0.22
ADP(10^{-16}moles/cell)	2.37±0.13	2.47±0.11
AMP(10^{-17}moles/cell)	3.03±0.16	3.42±0.29
AEC	0.97±0.001	0.97±0.001
NAD^+(10^{-16}moles/cell	7.59±0.53	7.86±0.50

[a]N=5(QO_2) and N=12 (adenylate phosphates and NAD^+)
[b]Mean ± standard error

*Note: All values in the presence of nicotinamide were only shown at 30 min prior to and during analysis; additionally, we observed no alterations in metabolic status after the nicotinamide treatment for 10 or 60 min.

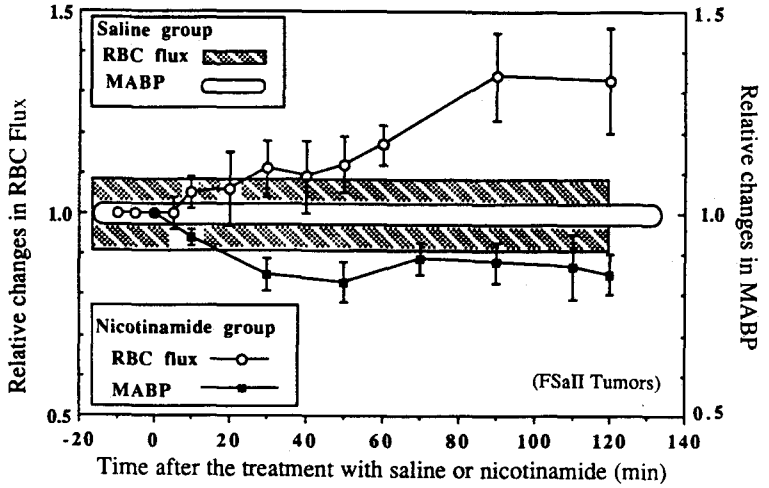

Fig. 1. *Changes in RBC flux and MABP after an i.p. injection of saline or nicotinamide in mice bearing*~150mm³ tumors. The cross-hatched region and the open-oval shaped region represent the fluctuation of RBC flux and MABP (N=5 mice) after the administration of 10 mg/kg isotonic saline (0.9% NaCl), respectively.

As is the case with cellular O_2 consumption, no significant changes either in the adenylate phosphate pools or energy charge were induced by nicotinamide treatment. Additionally, pretreatment for 10 or 60 min. resulted in no alterations in cell energy status or cellular NAD^+.

Fig. 1 shows the percent changes in relative blood perfusion (RBC flux) as well as in MABP in small-sized tumors(~150 mm³) as a function of time after an i.p. injection of 10 ml/kg of saline or 500 mg/kg of nicotinamide. In the saline-treated control group, RBC flux and MABP negligibly fluctuated for 2 h post-treatment ($p>.1$ in all cases). In the nicotinamide-treated group, the increase in RBC flux became significant by~35% above the initial value at 60-120 min. posttreatment ($p<.05$). However, MABP rapidly decreased within 10 min. after the nicotinamide treatment, reaching~15% reduction from the control values ($p<.05$) and then fluctuated slightly for 2 h post-treatmebt. This nicotinamide-induced reduction in MABP is consistent with previous publications[14-17]. Additionally, the mean MABP was 89±2 mmHg(N=22 mice, tumor volume:~100 mm³ to~500 mm³) when the mice in the saline-treated control group recovered from the anesthesia (3 h after recovery from anesthesia).

Fig. 2 shows the percent changes in RBC flux and MABP with various tumor sizes (from~250mm³ to~700 mm³) as a function of time after an i.p. injection of 500mg/kg of nicotinamide. Both MABP and RBC flux were simultaneously measured in all mice. In medium-sized tumors(~250 mm³) the RBC flux gradually improved, increasing by~75% above the initial value at 90 min post-treatment. MABP rapidly decreased within 10 min. after the nicotinamide treatment, reaching~15% reduction from the control values ($p<.05$) and then fluctuated slightly for 2 h post-treatment. Large-sized tumors (~500 mm³) also showed similar tendencies as the medium-sized tumors. RBC flux in larger tumors (~700 mm³), however, was not altered by nicotinamide. Additionally, there was no difference in changes in MABP after the nicotinamide treatment among any tumor-sized group.

To evaluate the possibility of water shift to the peritoneal cavity from the vascular compartment after treatment with nicotinamide, HCT was monitored after saline or nicotinamide treatment with the same injection volume (data not shown). HCT of mice bearing~150mm³ FSa II tumors was 48.5±0.5% immediately after an i.p. injection of saline at 10 ml/kg vs 49.1±0.4% im-

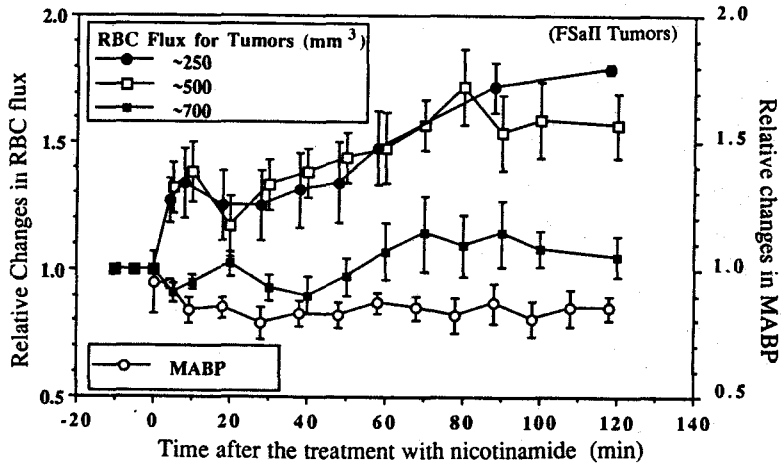

Fig 2. Changes in RBC flux after the nicotinamide treatment with various tumor volumes:~250mm³, 500mm³ and ~700mm³ tumors (N=5 mice in each group), and changes in MABP after the nicotinamide treatment with only~250mm³ tumors to avoid clutter in this figure. There was no difference in changes in MABP after the nicotinamide treatment among any tumor-sized group.

mediately after an i.p. injection of nicotinamide at 500 mg/kg. HCT fluctuated slightly during the 2 h post-treatment after nicotinamide injection ($p>.1$ in all cases).

DISCUSSION

In the present study the effect of nicotinamide on cellular O_2 utilization, cell energy status, cellular NAD^+ concentration and TBF has been examined in the FSaII murine tumor model. At the cellular level in vitro, changes in metabolic status or O_2 consumption were not observed for 30 min pretreatment at a nicotinamide concent-ration of ~4mM (=500 mg/kg). Similar results were observed with 10 and 60 min treatment (Table 2), and at~8 mM nicotinamide (data not shown). It, therefore, appears unlikely that nicotinamide may be expected to cause local changes in tissue oxygen status secondary to a decrease in O_2 consumption rates. Furthermore, an increase in pO_2 in the microenvironments of tumors is relying on an increase in O_2 availability (an increase in blood flow) and/or a reduction in O_2 consumption. It is well-known that O_2 consumption is determined by well-oxygenated tumor cells instead of hypoxic or anoxic cells because the hypoxic cells are deficient or devoid of O_2 ; therefore, nicotinamide would not inhibit O_2 consumption in vivo (or in vitro) if the cells or tissues in question were hypoxic or anoxic. Indeed, enhanced blood flow would be expected to reoxygenate hypoxic viable cells, and the rate of O_2 consumption by these cells would increase from 0 or low values, to normal values or to near normal values. If the values were only "normal or to near normal values" the hypoxic fraction would be reduced by a greater than expected percent.

We also observed that intracellular levels of ATP, ADP and AMP were not changed in the presence of nicotinamide. Additionally, no changes in intracellular levels of NAD^+ following nicotinamide pretreatment were observed in vitro. This does not preclude the possibility that in vivo, pretreatment with nicotinamide could lead to an increase in tumor cell NAD^+ levels; however, at the local cellular level, this does not occur. One possible explanation for the differences between our in vitro nicotinamide results and those of Kelleher and Vaupel[18] is that in vivo, nicotinamide is converted to nicotinic acid which is a known precursor of NAD^+[11]. This appears unlikely, however as O_2 consumption was unchanged in the presence of 4 mM nicotinic acid (data not

shown).

The lack of a significant change in O_2 consumption and metabolic status secondary to nicotinamide treatment contrasts with the increased TBF and pO_2[1] which occurs following nicotinamide treatment. This increased TBF appears to essentially elicit a complete reoxygenation of the 7-8 mm diameter FSa II tumor as evidenced by~30% reduction in TCD_{50}(unpublished data, Lee I, 1991). Also, the median pO_2 in these tumors increased from 3.0 mmHg to 6.1 mmHg[1].

Blood flow in solid tumors is heterogeneous both spatially and temporally[20]. These heterogeneities lead to chronic (diffusion-limited) and acute(perfusion-limited) hypoxia in tumors. Nicotinamide can increase TBF to alleviate chronic hypoxia, and/or can decrease fluctua-tions in TBF to alleviate acute hypoxia; however, TBF response is dependent on tumor type, size and nicotinamide dosage[1,4-6]. In some tumors there is a negligible increase in blood flow [7,17]. In work by Horsman et al., nicotinamide has been shown to decrease TCD_{50} by~20% in a C3H mammary carcinoma[19]. However, a significant decrease in energy status was observed without increasing blood perfusion[7]. Horsman et al.[7] suggested that nicotinamide may improve tumor oxygenation secondary to a reduction in O_2 consumption. Reduced energy status occurs secondary to reduced oxidative metabolism, especially if tumor glucose is limited(unpublished data, Gerweck et al., 1993).

The second goal of this study was to measure nicotinamide-induced O_2 availability as estimated by changes in relative TBF (RBC flux). Nicotinamide increased RBC flux, ranging from~35% in 150mm^3 tumors to~75% in 500mm^3 tumors (Figs.1 & 2). Interestingly, this increase was size-dependent; in the medium-sized tumors the increases in RBC flux was significantly more than that in the small sized tumors. This does not imply, however, that larger tumors have smaller hypoxic fractions after nicotinamide treatment. Although Lee and Song[1] observed that the magnitude of the increase in pO_2 by nicotinamide was relatively smaller in the small tumors than in the large tumors, the small tumors had higher pO_2 value than larger tumors. Based on our previous results on decrease in TIFP (tumor interstitial fluid pressure) following nicotinamide injection in a size-dependent manner[14], we speculate that nicotinamide may reduce the flow resistance in the tumor vasculature, and consequently incre-ase TBF.

Although nicotinamide appears to be a promising radiosensitizer for clinical trials due to its relatively low toxicity in humans[21], it is important to note that nicotinamide may not increase pO_2 or the radiation response of every tumor. For example, improved oxygenation (as measured with oxygen microelectrodes) in SCK murine tumors was not detected after nicotinamide treatment[22]. The median intratumor pO_2 in the control group and nicotinamide treated group was 4.6mmHg and 3.8 mmHg, respecti-vely. In addition, nicotinamide-induced radio-sensitization was not observed by a growth delay assay in SCK tumors.

The absence of changes in cell metabolic status, but increase in TBF is consistent with our studies which show that nicotinamide treatment leads to an alkalinization of tumor pH (un-published data, Lee I, 1993). An improved TBF may increase the removal of locally produced tumor lactic acid and other diffusible acid species[23]. For example, the mean pH(microelectrode) values before nicotinamide treatment in the small-sized (~150mm^3), medium-sized(~250mm^3) and large-sized tumor(~500mm^3) were 7.26, 7.01 and 6. 94, respectively. The pH values following nicotinamide treatment were 7.26, 7.16, and 7.16, respectively.

In conclusion, the results of this study indicate that a reduction in local tissue O_2 consumption is not a mechanism of improved tumor oxygenation by nicotinamide, in FSa II murine tumor model. The primary if not sole mechanism of tumor sensitization appears to be an increased local TBF as reported previously[1,2,4].

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=국문초록=

Nicotinamide에 의한 종양내 산소 분압의 증가에 있어서 혈류 또는 산소 소모의 역할

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조 문 준

Nicotinamide(NA)에 의한 종양내 산소 분압의 증가가 세포내 신진 대사의 변화 또는 산소 접근성의 변화에 기인하는지 규명하고자 NA의 세포내 산소 소모와 신진 대사에 미치는 영향을 다음과 같이 실험하여 보았다. 즉 시험관에서는 Adenylate Phosphates와 NAD^+의 변화를 동시에 생체에서는 혈류의 변화를 통하여 측정하였다. 세포 배양전 30분간 4mM(=500mg/kg) NA 처리시 세포내 산소 소모에는 영향이 없었다. 또는 4mM NA에서 세포내 Adenylate phophates와 NAD^+치의 변화도 없었다. 종양내 혈류의 변화(적혈구 흐름)로 생체내에서 NA가 산소의 접근성의 증가를 가져오는지 평가하였다. 레이저 도플러로 적혈구 흐름의 변화를 측정하였는데, 종양의 크기와 비례해서, 150mm^3 크기의 종양에서 적혈구 흐름이 35%증가하였으며 500mm^3 종양에서 75%증가하였다.

결론적으로 이상의 관찰에서 FSaII 생쥐 종양 모델에서 NA에 의한 종양내 산소 분압의 증가는 국소적 산소 소모의 감소에 의한 것이 아니며, 국소 종양내 혈류의 증가가 종양내 산소 분압 증가의 주 기전으로 사료된다.