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Hepatic Arginase - Nitric oxide imbalance: Impact of carcinogenesis and therapeutic effect of sodium channel blockage in an *in vivo* rat model

Hepatic Arginase - nitric oxide imbalance: Impact of carcinogenesis and therapeutic effect of sodium channel blockage in an *in vivo* rat model

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Abstract: Objective: Nitric oxide synthase and arginase are frequently antagonistic and interactive, although both use L-arginine as common substrate. Their balance is of potential functional importance. How the balance changes in cancer is unknown. Increasing evidence suggests that progression of carcinomas involves functional voltage-gated sodium channel (VGSC) activity.

Methods: The present study extended this study to liver and aimed to determine whether (i) DMBA carcinogenesis would affect the activities of arginase and NOS and (ii) treatment with Na-channel blocker RS100642 would ameliorate the impact of the carcinogen on the arginase-NOS balance.

Results: DMBA application significantly increased arginase activity and, correspondingly, the level of L-ornithine by 25–33%. In contrast, NOS activity decreased by 11%. Importantly, RS100642 treatment completely suppressed the effect on arginase.

Conclusion: It is concluded (i) that DMBA carcinogenesis changes the hepatic arginase-NOS balance, increasing the overall dominance of arginase and (ii) that VGSC inhibition has a protective effect on liver.

Keywords: Liver, Carcinogenesis, Arginase, Nitric oxide synthase, Sodium channels, RS100642

Özet: Amaç: Arjinaz ve nitrik oksit sentaz, arjinini ortak substrat olarak kullanmalarına rağmen sıklıkla antagonist ve etkileşim halindedirler. Aralarındaki denge potansiyel olarak fonksiyonel öneme sahiptir. Bu dengenin kanserde nasıl değiştiği henüz bilinmemektedir. Artan kanıtlar fonksiyonel voltaj kapılı sodyum kanal (VGSC) aktivitesinin kanser gelişiminde yer aldığını göstermektedir.

Metod: Bu çalışmada, karaciğerde (i) DMBA ile oluşturulan karsinojenezin arjinaz ve NOS aktivitelerini etkileyip etkilemediğinin (ii) RS100642 Na-kanal blokeri uygulanmasının arjinaz NOS dengesi üzerine karsinojen etkisini iyileştirip iyileştirmediğinin belirlenmesi amaçlandı.

Bulgular: DMBA uygulanması, arjinaz aktivitesini ve bununla bağlantılı olarak L-ornitin düzeylerini (%25–33) anlamlı şekilde artırdı. Buna karşın, NOS aktivitesi %11 oranında azaldı. Önemli bir bulgu olarak, RS100642 uygulanması arjinaz üzerine olan karsinojen etkisini tamamen baskıladı.

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Sonuç: Sonuç olarak; (i)DMBA ile oluşturulan karsinojenez, hepatik arjinaz-NOS balansını arjinazın tümüyle baskın hale geleceği şekilde artırarak değiştirmektedir, (ii) VGSC inhibisyonu karaciğer üzerinde koruyucu etkiye sahiptir.

Anahtar Kelimeler: Karaciğer, Karsinojenez, Arjinaz, Nitrik oksit sentaz, Sodyum kanalları, RS100642

1 Introduction

An association between arginine/arginase and cancer has been recognised for many years [1]. In mammalian cells, the semi-essential amino acid L-arginine is involved in protein synthesis but it also serves as a substrate to enzymes such as arginase, arginine decarboxylase, as well as nitric oxide synthase (NOS; E.C. 1.14.13.39 L-arginine,NADPH: oxygen oxidoreductase) and glycine transaminase. Arginase (E.C. 3.5.3.1 L-arginine amidinohydrolase) is a key enzyme responsible for nitrogen metabolism and from it forms urea and L-ornithine from arginine. In mammals, there are at least two distinct forms of arginase: Arginase I (AI) and Arginase II (AII). In liver, beside the predominant, cationic arginase AI (pI 9.3), the anionic arginase AII (pI 7.7) form also occurs [2]. It appears that AI plays a fundamental role in the last step of the urea cycle, whilst AII provides a supply of L-ornithine, a metabolite crucial for biosynthesis of glutamic acid, proline and polyamines. Since polyamines are vital for cellular proliferation, it is possible that the increased level of L-ornithine, due to the elevated arginase activity, is associated with cell proliferation and tumourigenesis [3].

Nitric oxide (NO) is synthesized from L-arginine by a family of three NOS isoenzymes: endothelial (eNOS), neuronal (nNOS) and inducible (iNOS). In particular, NO produced by iNOS is released for long periods by cells of the immune system, among others and can be cytostatic or cytotoxic to tumour cells [4]. Thus, L-arginine can generate opposing effects on cancer: promotion via production of precursors of polyamines and suppression via NO production, determined by the arginase-NOS imbalance (Figure 1). Similar competition occurs in cells of the immune system. Such competition may involve direct interaction(s) between arginase and NOS [5]. However, how the arginase-NOS balance is controlled, especially in cancer and *in vivo*, is not well understood.

Recent studies have demonstrated that increased voltage-sensitive sodium channels activity (VGSCs) plays a significant role in progression of various solid cancers [6,7]. Blocking VGSC activity with tetrodotoxin, siRNA or small-molecule inhibitors suppressed invasiveness *in vitro* [8,9]. More recently, the evidence for the role of VGSC activity in cancer progression has been extended to *in vivo* model [10].

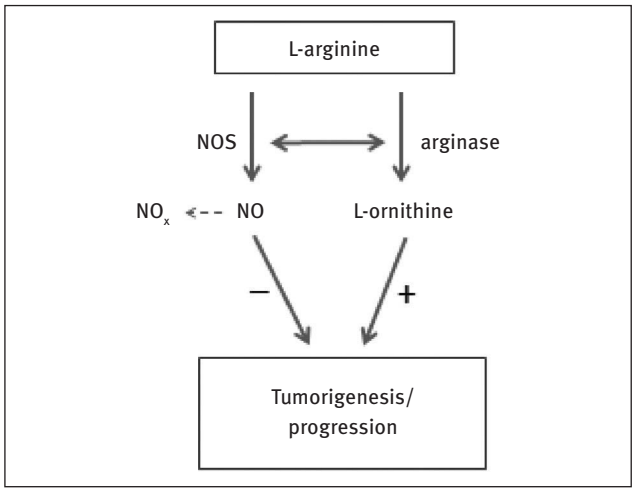


Figure 1: Schematic representation of the two main pathways through which L-arginine can affect tumourigenesis/tumour burden. Basically, L-arginine is the 'competitive' common substrate for arginase and nitric oxide synthase (NOS). L-arginine when metabolized by arginase yields L-ornithine which stimulates cell proliferation and thus promote tumour progression (denoted by "+"). L-arginine can also be metabolized by NOS to yield nitric oxide (NO) which can be cytotoxic (denoted by "-"). Strictly, at low doses, NO can also promote tumourigenesis. Here, the total amount of NO produced was measured as NO_x (NO_2+NO_3).

A common, chemically-induced *in vivo* model of carcinogenesis is that employing the polycyclic aromatic hydrocarbon, 7,12-dimethylbenz(a)anthracene - DMBA [11]. In a previous study, we have shown that the oxidative stress that accompanies the DMBA-induced carcinogenesis in mammary glands of rats can be the suppressed by the VGSC blocker, RS100642 [10]. The present study extends this work to liver samples from the same DMBA-treated rats. In particular, we aimed to determine (i) the effect of DMBA on the hepatic arginase-NOS balance and (ii) the possible impact on this of RS100642 co-treatment.

2 Materials and Methods

2.1 Animals

A colony of adult (180–210 g) female Sprague Dawley rats was obtained from Inonu University Laboratory Animal Research Centre, Malatya, Turkey. The animals

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were maintained and handled according to the regulations of the Institutional Ethic Committee. The present study involved 54 rats housed in a room maintained at 22°C with a 12-h light-dark cycle with free access to foods. Rats were randomized into three groups (18 rats per group): Group I, served as control, were intraperitonally administrated with a single dose of 0.5 ml corn oil. Rats in groups II (DMBA) and III (DMBA+RS100642) were intraperitonally administrated with a single dose (20 mg/kg b.w.) of DMBA (Sigma Chemicals). DMBA was dissolved in corn oil and given in a volume of 0.5 ml [10]. The mammary glands were palpated weekly for tumour appearance. The first tumour was detected ~120 days after the DMBA administration. After 150 days, the rats in Group III were intravenously administered with RS100642 (0.25 mg/kg b.w. dissolved in 250 µl of 0.9% NaCl), once a week for four weeks (total dose:1 mg/kg b.w., at the same time the rats in Group I and II were i.v. administered with 250 µl of 0.9% NaCl, once a week for four weeks. Rats in all groups were sacrificed by cervical dislocation under ether anaesthesia at the end of 178 days. Livers were dissected, washed in 0.9% NaCl and frozen at -80°C.

2.2 Preparation of tissue homogenates

The frozen whole liver tissues were divided into two portions. One portion was stored for measurements of arginase activity and L-ornithine level. The other was cut into small pieces, homogenized in PBS solution (1:5 w:v) and centrifuged at 13.000 g for 15 min at 5°C (5417R, Eppendorf Aktiengesellschaft, Hamburg, Germany). The supernatant was separated and stored at −80°C for measurements of the metabolic by-products of NO (NO_x) in total (NO_2+NO_3).

2.3 Tissue arginase activity and L-ornithine levels

The frozen tissues were homogenized in 10 volumes of cold 0.05 M Tris/HCl buffer (pH 8.05). Samples then were centrifuged at 11.000 g for 20 minutes at 4°C. For the determination of tissue arginase activity and L-ornithine level, the methods of Geyer & Dabich [12] and Chinard [13] were used, respectively. One unit of arginase activity was expressed as the amount of enzyme catalysing the formation of 1 µmol of urea in an hour at 37°C (Unit/mg protein) and L-ornithine levels were expressed as µmol/mg protein.

2.4 Nitric oxide metabolite assay

The level of NO_x (mainly NO_2+NO_3) was measured by the method of Sastry *et al*. [14]. A calibration standard involving potassium nitrate was used to calculate the total concentrations of nitrates, which were expressed as µmol/mg protein.

2.5 Data analysis

The results are expressed as mean ± standard error. Statistical analysis was carried out using the SPSS 16.0 statistical program (SPSS Inc., Chicago, IL, USA). Data presented a normal distribution with a One-sample Kolmogorov-Smirnov test. The One-way ANOVA and LSD Post Hoc test techniques were performed to test the differences between groups; $p<0.05$ was considered statistically significant.

3 Results

The raw data obtained and their statistical analyses are shown in Table 1. Data are plotted on a relative scale for direct comparison of the three groups in Figure 2.

In the DMBA group (II), arginase activity and, correspondingly, the L-ornithine level were significantly higher (by 30 and 33%, respectively) compared with the controls (group I) ($p<0.05$). Both these effects were suppressed completely by the RS100642 co-treatment. There was no difference in the L-ornithine levels between the control and the DMBA+RS100642 groups ($p>0.05$). In the case of arginase activity, in fact, the value for the DMBA+RS100642 group (III), fell below the control value by 20% on average. This implied that RS100642 also suppressed the basal level of arginase activity that may occur in rat liver. Thus, the NO_x level in the DMBA-treated rats was significantly lower than the control by an average of 10% ($p<0.05$). Treatment with RS100642 partially (by some 5%) suppressed this decrease. Although the latter was not statistically significant, the quantitative shift resulted in the disappearance of the difference in the NO_x levels between the DMBA+RS100642 and the control groups ($p>0.05$).

4 Discussion

Polyamines plays critical role in cellular proliferation in tumourgenesis and their levels associated with imbal-

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Table 1: Effects of DMBA treatment and co-treatment with RS100642 on arginase activity and L-ornithine and NO_x levels in rat liver tissue. Raw data given as mean±SEM. n is the number of animals in each of the three groups. Note that four animals died in groups II and III.

Group (n)	Arginase (U/mg protein)	L-Ornithine (μmol/mg protein)	NO_x (μmol/mg protein)
I. Control (18)	52.46±2.48	0.12±0.01	10.06±0.35
II. DMBA (14)	68.52±3.80	0.16±0.01	8.52±0.36
III. DMBA+RS100642 (14)	42.26±4.07	0.13±0.01	9.66±0.76
		P values	
Group comparisons	**Arginase**	**L-Ornithine**	**NO_x**
I versus II	0.01*	0.001*	0.032*
I versus III	0.034*	0.347	0.566
II versus III	<0.0001*	0.003*	0.136

*Statistically significant differences ($p<0.05$).

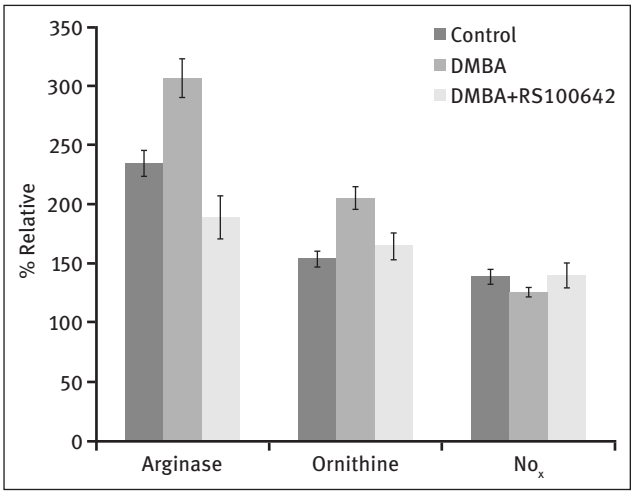


Figure 2: Effects of DMBA and DMBA+RS100642 treatments on hepatic NOS-Arginase system parameters (arginase activity, and levels of L-ornithine and NO_x). Normalized average percentages are shown relative to each respective control group with the lowest value in each set fixed as 100%. The shading of the columns indicates the different treatments as follows: controls/without treatment (white); treatment with DMBA (black); and DMBA+RS100642 (grey). Each bar represents mean±SEM (n≥14).

ance between arginase and NOS. Wang *et al*. also reported high ornithine decarboxylase (ODC) activity and L-ornithine level in skin carcinogenesis induced by DMBA and 12-O-tetradecanoylphorbol-13-acetate [15]. Correspondingly, decreased L-ornithine levels and inhibition of ODC activity suppressed tumour cell proliferation [16].

Under identical conditions, the DMBA treatment had an opposite, inhibitory effect on NOS activity. Thus, the NO_x level in the DMBA-treated rats was significantly lower than the control. Treatment with RS100642 slightly suppressed this decrease. As in the case of arginase, RS100642 suppressed the effect of DMBA treatment on NOS activity. The involvement of NO in carcinogenesis is complex [4]. Here, the DMBA treatment was found here to decrease the level of NO_x, whilst it enhanced expression of iNOS in hamster buccal-pouch carcinoma and high levels of NO caused death of tumour cells [17]. It is likely that the role of NO in tumorigenesis is concentration-dependent whereby low concentrations promote tumour cell proliferation whilst relatively higher concentrations are inhibitory [4]. A further complexity is the potential competition between AII and NOS, but the arginase pathway appeared more dominant [5,18]. Such 'dominance' would both accelerate tumour development (e.g. via the proliferative effect of polyamines) and protect tumour cells from apoptosis by reducing NO production. However, it is not clear how AII (a mitochondrial enzyme) might compete with NOS for intracellular L-arginine. One possibility is that an increase in mitochondrial L-arginine degradation by AII results in enhanced transport of L-arginine from cytosol into mitochondria, thereby reducing the availability of cytosolic L-arginine for NO synthesis.

Importantly, following treatment with the novel VGSC blocker RS100642, the DMBA-induced changes in arginase activity/L-ornithine level and NO production were eliminated. We showed previously that the RS100642-treated rats lived 26% longer [10]. The mechanism(s) through which VGSC activity could change the arginase-NOS imbalance is not clear at present but the following is one possible scenario: VGSC activity (especially the 'persistent current' component) would raise the intracellular Na^+ concentration which could then slow down (or even reverse) Na^+-Ca^{2+} exchange and thus lead to a rise in intracellular Ca^{2+} concentration [19]. The latter can activate a number of protein kinases which may enhance arginase activity under resting conditions. This could also determine the nature of the NOS(s) involved, iNOS being Ca^{2+} insensitive. Additional

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effects may occur as a result of acidification of intracellular pH by the slowing down of Na^+-H^+ exchange via NHE1 and arginase is well known to be pH dependent [20].

5 Conclusions

Carcinogenesis in rats changes the hepatic arginase-NOS balance in favour of the former and this is controlled, at least in part, by VGSC activity. Thus, VGSC inhibition can counteract the adverse biochemical changes that occur during tumour development/progression and promote survival, consistent with previous *in vitro* and *in vivo* findings. More broadly, it seems possible to reverse the metabolic reprogramming that occurs during carcinogenesis using ion channel modulators.

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Conflict of interest: None declared.

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