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Original article

Is tryptophan catabolism increased under indoleamine 2,3 dioxygenase activity during chronic lung inflammation in pigs?

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Abstract – In a preliminary study we observed that piglets suffering from chronic lung inflammation induced by an intravenous injection of complete Freund adjuvant showed a marked decrease in plasma tryptophan (Trp) concentration suggesting increased Trp utilisation. During the inflammatory process, a cytokine-induced enzyme called indoleamine 2,3-dioxygenase (IDO) has been shown to catabolise Trp into kynurenine (Kyn). Yet, during inflammation, increased Trp catabolism may decrease Trp availability for other functions such as growth. This metabolic pathway has never been studied in pigs. So, the objectives of this study were to measure IDO activity in pigs and to determine if the decrease in plasma Trp concentrations previously observed in piglets suffering from chronic lung inflammation could be explained by the induction of IDO activity. In order to do so, we compared IDO activity measured in the tracheo-bronchial lymph nodes and in the lungs of 7 piglets, injected with complete Freund adjuvant (CFA), to 7 pair-fed littermate healthy controls. Blood samples were taken at 0, 2, 5, 7 and 10 days following CFA injection in order to measure plasma Trp, Kyn and haptoglobin concentrations. Indoleamine 2,3-dioygenase activity in the tracheo-bronchial lymph nodes ($P < 0.05$), in the lungs ($P < 0.07$) and plasma haptoglobin ($P < 0.01$) were higher in pigs with lung inflammation than in the controls. Plasma Trp and Kyn were not significantly affected by CFA injection. Our data showed that IDO is activated under chronic lung inflammation in pigs. The impact of IDO activation on plasma Trp concentration and its availability is discussed according to the amount of Trp provided by the diet.

tryptophan / indoleamine 2,3-dioxygenase / inflammation

1. INTRODUCTION

When pigs are exposed to high antigen pressure, growth is impacted even if pigs are not displaying serious clinical signs of disease. For instance, the high prevalence of subclinical chronic pneumonia is confirmed by the frequent occurrence of lung lesions at slaughter [1]. In such conditions, the decrease in growth is explained by a

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decrease in feed consumption but also by the diversion of nutrients from growth towards tissues and cells involved in immune responses. Increased utilisation of some specific amino acids induced by immune system activation is not necessarily balanced by dietary supplies and may modify amino acid requirements [2]. In a preliminary study we observed that piglets suffering from non infectious chronic lung inflammation had significant lower plasma tryptophan (Trp) concentration than pair-fed healthy piglets suggesting increased Trp utilisation for other processes than growth [3]. In various states of immune activation in humans, macaques, rabbits, mice and rats, the depletion of free serum Trp has been related to increased Trp degradation under activation of indoleamine 2, 3 dioxygenase (IDO, EC 1.13.11.42) [4–6]. Indoleamine 2, 3 dioxygenase converts Trp into N-formylkynurenine which is further catabolised to kynurenine (Kyn). This is the first step of a metabolic pathway that ends at niacin metabolite production. This enzyme is stimulated by pro-inflammatory cytokines, especially interferon-γ (IFN-γ) [7, 8]. IDO has a large tissue distribution including the lungs, gut, spleen but not in the liver [9–11]. Our interest to this enzyme arises because the induction of the IDO pathway may be a mechanism that limits the availability of the essential amino acid Trp for protein accretion during the inflammatory process. Moreover, the IDO pathway may play crucial roles in the regulation of the immune and inflammatory responses [12–14]. We also question also the role of Trp in body defenses. Yet, in pigs, data on IDO activation and functions are non-existent. Therefore, the objective of this study was to measure IDO activity in pigs with chronic lung inflammation. Secondly, we aimed at determining if the decrease in plasma Trp concentration previously observed in piglets suffering from chronic lung inflammation could be explained by the induction of IDO activity and thus an increased Trp catabolism.

2. MATERIALS AND METHODS

2.1. Animals and experimental design

All procedures were performed according to current legislation on animal experimentation in France (authorisation No. 7719 delivered by the French Ministry of Agriculture and Fisheries). Twenty days post-weaning, seven pairs of littermate piglets were selected on the basis of their body weight (13.5 ± 1.3 kg). Within a pair, the weight difference was always lower than 1 kg. Under general anaesthesia induced with ketamine (Imalgene 100, Merial, France) and maintained with 2 to 5% halothane (Belamont, France) in oxygen, an indwelling silicone catheter (0.76 × 1.65 mm, Ercelab Vermed; ref. 48175) was implanted through a collateral vein in the right external jugular vein. The catheter tube was placed under the skin, externalised on the dorsum of the neck and put in a bag sutured to the skin. The catheters were flushed daily with 10 mL of sterile saline solution containing 2.5 mL of heparin (5000 $U \cdot mL^{-1}$). The pigs were housed in individual cages in an environmentally controlled building with alternate lighting and room temperature was maintained at 26 °C.

Nine days after surgery and following an overnight fast, one piglet per pair was slowly injected with 3 mL of Complete Freund adjuvant (Sigma Aldrich; catalog F-5881) in 10 mL of sterile saline solution (INFL). Complete Freund adjuvant (CFA) is a mineral oil containing killed *Mycobacterium tuberculosis* cells. Its intravenous injection reproduced interstitial pneumonia lesions [3, 15]. The seven other piglets were intravenously injected with the same volume of sterile saline and constituted the control group (CON).

The pigs were fed with a standard phase II post-weaning diet twice a day (the composition is given in Table I). This diet was formulated to provide 12 g of lysine per kg of diet (10.9 $g \cdot kg^{-1}$ digestible lysine) and 2.4 g

Table I. Composition of the diet.

Ingredients	% as fed basis
Ground wheat	23.2
Ground corn	28.0
Ground barley	17.2
Soybean meal	26.6
Vegetable oil	0.4
Calcium carbonate	0.64
Dicalcium phosphate	1.62
Sea salt	0.4
L-lysine	0.44
DL-methionine	0.10
L-threonine	3.3
L-tryptophan 10%	0.09
Phytase	0.2
Salocine 20	0.2
Vitamin – trace mineral mix[a]	0.5

[a] Supplied per kg of diet: vit A, 10 000 IU; vit D3, 2 000 IU; vit E, 20 mg; vit K3 (menadione), 2 mg; vit B1 thiamin, 2 mg; vit B2 riboflavin, 5 mg; vit B3 niacin, 20 mg, vit B5 panthotenic acid, 10 mg; vit B6 pyridoxine, 5 mg; vit B8 biotin, 0.2 mg; vit B9 folic acid, 1 mg; vit B12 cyanocobalamin, 0.03; choline, 500 mg; vit C ascorbic acid, 40 mg; (mg) Fe: 100; Cu: 20; Mn: 40; Zn: 100; Co: 1; I: 0.6; Se: 0.3.

of Trp·kg^{-1} of diet (2.1 g·kg^{-1} of digestible Trp). However, the analysis of dietary amino acid composition gave higher quantities of total Trp, 3.2 g·kg^{-1}, and lysine, 12.4 g·kg^{-1}, than it was expected by the formulation. Within a litter, the piglets were pair-fed in order to avoid confounding effects of feed intake and inflammation on plasma Trp concentration. Within a litter, the feed intake of the INFL piglet was measured 1 h after food distribution and the same quantity of food was then offered to the CON littermate. The amount of food allocated was limited to 40 g·kg^{-1} BW in order to ensure that pair-fed CON pigs would always eat all the given food. All pigs had free access to water.

2.2. Blood and tissue analyses

After an overnight fast, blood samples were taken 0, 2, 4, 7 and 10 days after CFA injection. Plasma Trp and Kyn concentrations were measured by an HPLC method [16]. Plasma haptoglobin concentrations were quantified by a colorimetric reaction using commercial kits (Phase Haptoglobin Colorimetric Assay, Tridelta, Ireland). Haptoglobin is a major positive acute phase protein in pigs used as an indicator of inflammation. Plasma haptoglobin concentration is usually increased under several situations of inflammation [3, 17, 18]. At day 10, the piglets were slaughtered. Because lungs are the target tissue of CFA challenge, we decided to measure IDO activity in tracheobronchial lymph nodes and lungs. These tissues were immediately removed, weighed and kept at –80 °C until analysis. The tissues were ground in liquid nitrogen and homogenised in a cold potassium buffer (KH_2PO_4/Na_2HPO_4,$2H_2O$, 20 mM and KCl, 140 mM, pH 7.0) with a polytron homogeniser (Kinematica, Suisse) at 9 500 rotations per minute. The homogenates were centrifuged at 14 000 *g* for 30 min at 4 °C and IDO activity was determined in the supernatant according to the method described for mice by Lestage et al. [19]. Briefly, the tissue homogenate (200 µL) was mixed with a reaction medium (800 µL) containing 400 µM Trp, 20 mM ascorbic acid, 20 µM methylene blue and 100 µg beef liver catalase (Roche, 106810). The mixture was then incubated during 3 h in a bath at 37 °C to produce N-formylkynurenine from Trp. The reaction was stopped with the addition of 200 µL trichloroacetic acid (30%) and further incubated at 50 °C for 30 min to hydrolyse N-formylkynurenine produced by IDO to Kyn. The reaction mixture was then centrifuged for 10 min at 13 000 *g* to remove sediment. Then, the supernatant was purified on MICROCON membranes (Millipore, USA) by centrifugation for 45 min at 3000 *g*. The quantity of formed Kyn was determined by HPLC as described above. To calculate the specific activity of IDO, the

Table II. Body weight (BW), daily weight gain, liver and lung weight in pair fed pigs injected i.v. with saline solution (CON) or challenged with 3 mL of complete Freund adjuvant (INFL). Data are least squares means calculated from the whole experimental period for BW and ADG ± SEM, $n = 7$ in each treatment group. Liver and lung weight were determined at slaughter 10 days after complete Freund adjuvant injection.

	INFL	CON	SEM	C[1]
Animal performance				
BW kg	15.56	15.56	0.17	NS
ADG g·day^{-1}	363.6	372.5	10.4	NS
Tissue weight (W)				
Liver W g·kg^{-1} BW	23.8	21.5	0.5	**
Lung W g·kg^{-1} BW	19.0	11.1	0.9	***

[1] C indicated the challenge effect, ** and *** indicated significant effects of challenge respectively for $P < 0.01$ and $P < 0.001$. NS indicated non significant effect of challenge injection.

protein concentrations in the homogenate were determined according to the colorimetric method with the Bicinchonic acid detergent compatible assay (Interchim, France).

2.3. Calculations and statistical analysis

Data were analysed with the GLM procedure of SAS (SAS Inst. Inc., Cary, NC). The model included challenge (INFL vs. CON), pair, time (days) and their interactions. The effect of challenge was tested using challenge × pair as the error term. For plasma variables such as haptoglobin, Trp and Kyn, the effects of time and its first order interactions with pair and challenge were tested with the residual error pair × time × challenge.

3. RESULTS

3.1. Animal clinical observations and weights

Following CFA injection, the pigs became lethargic and rapidly showed increased respiratory rhythm. After two days, INFL piglets seemed to recover. In INFL pigs, we did not record a significant decrease in food consumption relative to the amount of food offered. In addition, CFA had no effect on the body weight and the daily weight gain of the piglets (Tab. II). At slaughter, macroscopic granulous pulmonary lesions were observed in INFL pigs. In addition, lymph nodes were increased in size in INFL pigs compared to CON pigs. Average lung and liver weights relative to BW were significantly higher ($P < 0.05$) in INFL than in CON pigs (Tab. II).

Rectal temperatures were higher in INFL ($P < 0.05$) than CON pigs regardless of time. One and two days following CFA injection, INFL pigs had higher average rectal temperatures than CON pigs ($P < 0.01$ and $P < 0.05$ respectively, Fig. 1). From the third day, rectal temperatures of INFL pigs regained those of CON pigs until days 5 and 6 when temperatures were higher ($P < 0.05$ and $P < 0.01$) in INFL than in CON pigs. At the end of the second week of the experiment, rectal temperatures were not significantly different between the two groups of pigs.

3.2. Plasma haptoglobin

Plasma haptoglobin concentrations are illustrated in Figure 2. The challenge ($P < 0.05$) and the interaction challenge × time

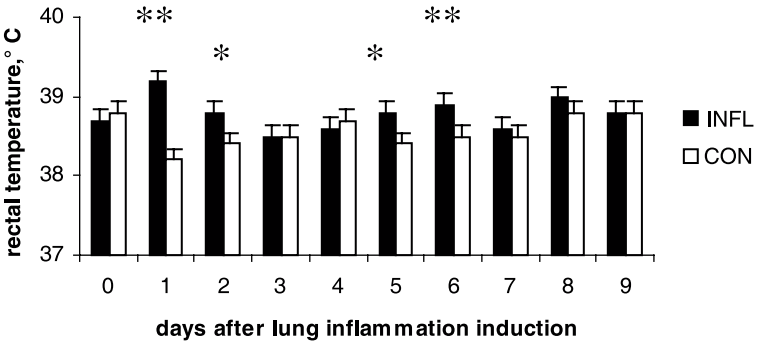

Figure 1. Rectal temperature in pigs injected i.v. with saline (CON) or 3 mL of complete Freund adjuvant (INFL). Data are least squares means ± SEM; $n = 7$ in each treatment group. Symbols denote significant differences between treatments (* $P < 0.05$, ** $P < 0.01$).

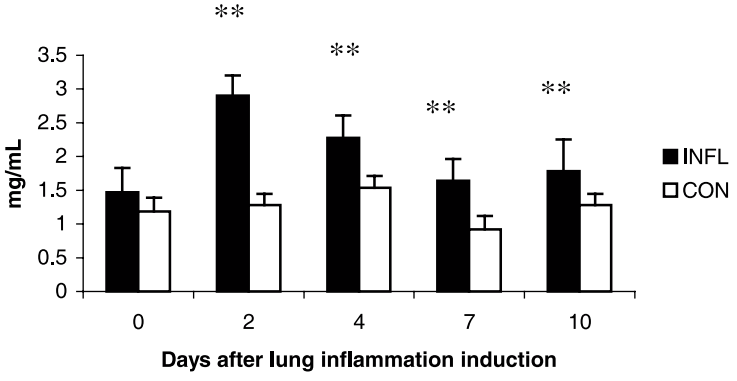

Figure 2. Plasma haptoglobin concentrations in pigs injected i.v. with saline (CON) or 3 mL of complete Freund adjuvant (INFL). Data are least squares means ± SEM, $n = 7$ in each treatment group. Symbols denote significant differences between treatments (** $P < 0.01$).

($P < 0.05$) effects on plasma haptoglobin concentration were significant. Before CFA injection, haptoglobin concentrations were not different ($P > 0.05$) between INFL and CON pigs. CFA injection induced an increase in plasma haptoglobin concentration throughout the study. Two days after CFA injection, plasma haptoglobin concentration in INFL pigs was more than two times that of CON pigs (respectively 3 mg·mL^{-1} vs. 1.4 mg·mL^{-1}). At day 10, plasma haptoglobin concentrations were still higher ($P < 0.01$) in the INFL pigs than in the CON pigs.

3.3. Plasma Trp, Kyn and IDO activity

IDO activity measured in tracheo-bronchial lymph nodes was significantly higher ($P < 0.05$) in INFL than in CON pigs (Fig. 3). In the lungs, because IDO was not induced in two piglets from the INFL group, IDO activity tended to be higher ($P < 0.07$) in

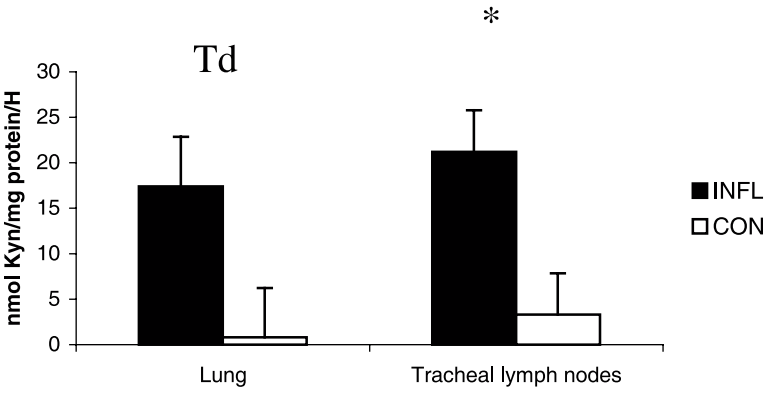

Figure 3. Indoleamine 2,3 dioxygenase (IDO) activity in the lung and tracheo-bronchial lymph nodes in healthy pair-fed pigs (CON) or in pigs injected i.v. with 3 mL of complete Freund adjuvant (INFL). Data are least squares means ± SEM, $n = 7$ in each treatment group for lung data and $n = 6$ for each treatment group for tracheal lymph-nodes. Td indicates $P < 0.1$ and * indicates $P < 0.05$.

INFL pigs than in CON pigs (Fig. 3). Neither challenge nor challenge × time interaction effects were significant in plasma Trp and Kyn concentrations. Therefore, only the average values of Trp and Kyn concentrations for CON and INFL are represented (Tab. III).

4. DISCUSSION

INFL pigs had greater plasma haptoglobin concentrations throughout the study, higher liver and lung weights relative to BW and they displayed lung lesions at slaughter confirming the inflammation was set up. We measured and observed the induction of DO activity following chronic lung inflammation for the first time in pigs. The higher IDO activity measured in tracheal lymph nodes and in the lungs of the INFL group showed that Trp catabolism may increase under inflammatory response in pigs. Nevertheless, the reason why 2 INFL pigs out of 7 did not respond by an increase in IDO activity in the lungs despite IDO activity being increased in tracheo-bronchial lymph nodes remains unclear. Indeed, for these two piglets, the lung weight expressed relative to body weight was significantly

Table III. Plasma tryptophan (Trp) and kynurenine (Kyn) concentrations in healthy pair-fed pigs (CON) or in pigs injected i.v. with 3 mL of complete Freund adjuvant (INFL) measured after an overnight fasting. Data are least squares means calculated from all the time points (0, 2, 5, 7 and 10 days after complete Freund adjuvant injection) of the experimental period ± SEM, $n = 7$ in each treatment group.

	INFL	CON	SEM	C[1]
Trp (nmol·mL^{-1})	36.79	38.77	2.91	NS
Kyn (nmol·mL^{-1})	1.08	1.12	0.05	NS

[1] C indicated the INFL challenge effect, NS indicated that P-value > 0.05 whereas the sign * indicated significant effects of INFL challenge $P < 0.05$.

higher than for their respective pair-fed littermate. The increase in IDO activity may not have occurred, or, alternatively, it had occurred at a different time from slaughter. In the lungs, we observed a 22 times increase in IDO activity between INFL and CON pigs which was in accordance with the results obtained in mice injected with pokeweed mitogen [4] or treated with LPS [8] (respectively 20 times and 18 times increase in IDO activity compared to controls). In macaques infected with type D-retrovirus [20], IDO activity in the lungs was increased only 5 times compared to healthy monkeys. The roles and functions of IDO under pathological situations and inflammation states have not yet been elucidated. The induction of IDO activity has been first studied for its potential antimicrobial function [12, 21, 22]. The most commonly suggested mechanism is the ability of IDO to reduce the availability of Trp that is an essential nutrient for the development of the pathogen. Secondly, IDO induction has been envisaged as a free-radical protector system. This latter role may be attributable directly to IDO activation that removes superoxyde radicals using them both as the substrate and cofactor [23] and also indirectly via the production of metabolites such as 3-hydroxy anthranilic acid and 3-hydroxy Kyn that may function as free-radical scavengers and antioxidants [13, 24]. Recently, studies have stressed the fact that IDO activity may have other functions than combatting infections and their consequences. This idea came from the observation that, in healthy subjects, IDO activity is expressed in several tissues such as the epididyme, placenta, spleen, thymus and lymph nodes. In the present study, CON pigs displayed a basal level of IDO activity in tracheo bronchial lymph nodes suggesting a potential role of IDO in this tissue independently of inflammatory response. This basal activity may be related to immune tolerance processes induced by suppressing T cell activation and proliferation [25–29].

Our previous experiment [3] showed that pigs suffering from chronic lung inflammation induced by CFA injection had lower plasma Trp concentrations than pair-fed healthy pigs. In several species, humans, macaques, rabbits or mice, IDO induction by infections, viruses, or inflammatory diseases, was associated with both a decreased plasma Trp and increased plasma Kyn concentrations [4–6]. Yet, in the present study it is surprising that, despite the induction of IDO activity, neither plasma Trp concentration nor Kyn were affected by chronic lung inflammation. Thus the lack of response of plasma Trp concentration is intriguing and needs to be discussed. In healthy subjects, the hepatic enzyme tryptophan 2,3-dioxygenase (TDO) is the main Trp-oxidative enzyme and plays a major role in controlling the serum Trp in response to dietary intake. Under immune system activation some authors have shown that its activity generally decreases when IDO activity increases. Consequently, it could be hypothesised that the balance between TDO and IDO may have occurred in order to maintain Trp availability for the tissue during inflammatory response. However, in our previous experiment we noticed a decrease in plasma Trp concentrations suggesting that this balance can be broken during inflammatory response [3]. In the present study, free Trp was added to the diet which was not the case in our previous experiment [3]. Furthermore, we recorded less food refusal relative to allocated amounts in the present study while food consumption was significantly decreased in INFL pigs in our previous one. Therefore, we hypothesised that Trp was probably not limiting in the present experiment in contrast to the previous experiment. In other words, even if Trp catabolism was increased through the IDO pathway, its plasma level could have been maintained by the Trp dietary supply. This idea is supported by further calculations we made only with data from the INFL pigs group. In INFL pigs, plasma Trp concentrations were negatively correlated with IDO activity measured in tracheal lymph nodes and in the lungs (respectively $P < 0.05$, correlation coefficient –0.89 and $P < 0.055$, coefficient

–0.75). These results suggest that IDO activity may be responsible in part for the increased Trp utilisation in pigs with inflammatory response. From a practical point of view, we question the quantitative impact of IDO activity on Trp metabolism. Is IDO activity of sufficient magnitude to divert the essential amino acid Trp from protein accretion? Also, plasma amino acid profiles might not represent a fully satisfactory marker of amino acid metabolism. The pool size of a plasma molecule is determined by the balance between its supply represented by protein breakdown and the diet, and its utilisation, catabolism or incorporation in larger molecules. Techniques using tracer infusion would be relevant to determine the contribution of the IDO pathway on Trp fluxes especially during immune system activation.

It is surprising that Kyn did not accumulate in the plasma of INFL pigs in spite of increased IDO activity. This may be explained by the fact that Kyn can be hydrolysed in other active metabolites such as 3-hydroxy Kyn, 3-hydroxy anthranilic acid, picolinic acid or quinolinic acid that may play various roles during inflammatory response as suggested above. Twenty-four hours after systemic pokeweed mitogen administration to mice, not only plasma concentrations of Kyn were increased but also those of 3-hydroxyKyn and quinolinic acid which are other Trp metabolites along the Kyn pathway [4].

In conclusion, we showed that IDO activity is induced in lung and lymph nodes of pigs with chronic lung inflammation. Thus inflammation may reduce Trp availability by increasing Trp catabolism. However this induction is not associated with a decrease in Trp and an increase in Kyn in the plasma. In the present experiment the lack of response of plasma Trp could be explained by the level of dietary Trp. Considering plasma Trp concentration as an indicator of Trp availability for growth and other normal physiological processes, Trp may become limiting if the supply of Trp in the diet is not sufficient to support increased utilisation caused by inflammation. Therefore, this work will be completed by a study on Trp metabolism in pigs with chronic lung inflammation receiving different levels of Trp in the diet.

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