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Surplus arginine reduced lipopolysaccharide induced transcription of proinflammatory genes in Atlantic salmon head kidney cells

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ABSTRACT

In aquaculture production, studies of salmon health and interaction between pathogens and nutrition are of high importance. This study aimed to compare genes and pathways involved in salmon head kidney cells and liver cells, isolated from the same fish, towards polyinosinic acid: polycytidylic acid (poly I:C) and lipopolysaccharide (LPS), with and without addition of surplus arginine. Selected transcriptional responses of genes involved in inflammation, polyamine synthesis, oxidation and apoptosis were elucidated.

For the genes related to inflammation, *viperin*, *Mx* and *Toll like receptor 3* (*TLR3),* transcription were significantly upregulated by poly I:C in head kidney cells, while *viperin* was upregulated in liver cells. Surplus arginine did not affect poly I:C induced responses with the exception of reducing poly I:C induced *Mx* transcription in head kidney cells. Gene transcription of *Interleukin 1β* (*IL-1β*), *Interleukin-8* (*IL-8*) and *cyclooxygenase 2* (*Cox2*) were elevated during LPS treatment in all liver and head kidney cell cultures. In addition, LPS induced significantly, *CD83* transcription in liver cells and *TNF-α* transcription in head kidney cells. Surplus arginine significantly reduced *IL-8, Cox2 and TNF-α* transcription in head kidney cells. LPS upregulated arginase in head kidney cells while poly I:C upregulated *S-adenosyl methionine decarboxylase* (*SAMdc*) transcription in liver cells. This suggests that LPS and poly I:C modulates genes involved in polyamine synthesis. In addition, in head kidney cells, surplus arginine, when cultured together with LPS, increased the transcription of *ornithine decarboxylase* (*ODC*) the limiting enzyme of polyamine synthesis. The genes involved with oxidation and apoptosis were not affect by any of the treatments in liver cells, while LPS decreased *caspase 3* transcription in head kidney cells. In liver cells, protein expression of catalase was reduced by surplus arginine alone and when challenged with poly I:C. Both liver cells and head kidney cells isolated from the same individual fish responded to LPS and poly I:C, depending on the gene analyzed. Additionally, arginine could modulate transcription of pro-inflammatory genes induced by LPS in salmon immune cells, thus affecting salmon immunity.

1. Introduction

Fish in both nature and aquaculture are exposed to stressful conditions, which may reduce growth and impair fish health. Arginine, an indispensable amino acid for fish [1] plays important roles in regulation of metabolism and immunity in animals [2].

Several studies indicated that arginine deficiency reduced the growth performance, decreased protein retention, reduced cellular polyamine concentrations and therefore the survival of aquatic organisms when challenged [3–7]. On the other hand, arginine supplementation above established requirement increased the growth and survival of several species of fish [6,8–11]. Metabolically, arginine is an essential precursor for the synthesis of polyamines (PA), nitric oxide (NO), creatinine and urea [2]. Arginase carries out the hydrolytic cleavage of arginine releasing ornithine and urea in the hepatocyte. Ornithine is the precursor of PA converted by the enzyme ornithine decarboxylase (ODC). The PA synthesis requires amino propyl groups that are released by S-adenosyl methionine (SAM). Due to the supply of propyl groups, SAM is descarboxylated by the enzyme SAM-decarboxylase (SAMdc) [12].

During PA turnover, spermidine and spermine are acetylated by the enzyme spermidine/spermine-N1-acetiltransferase (SSAT) [10]. PA acetylation consumes ATP and AcCoA, which might deplete energy from the cells [13,14]. The concentration of PA and NO in the cells are involved in the inflammatory processes and in the survival of the cells [15].

The head kidney in teleost has the hematopoietic function equivalent to bone marrow of mammals [16]. Usually the immunity of these

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animals is evaluated through this lymphoid organ, but the metabolic organ (liver) of fish is also affected when exposed to pathogenic and non-pathogenic bacteria, viruses, nutrients and contaminants. In order to cope with environmental challenges, the liver cells have to extract nutrients, residues or chemicals from the blood, trying to maintain the balance for the proper functioning of the organism [17,18].

LPS, the main component of external membrane of gram-negative bacteria is recognized by the innate immune system as a highly conserved pathogen coded molecular structure termed pathogen-associated molecular patterns (PAMPs). The PAMPs are recognized by pattern recognizing receptors (PRRs), and are therefore responsible for the first line of cellular innate immunity defense [19]. In mammals, Toll-like receptors (TLRs) are the best understood PAMPs that bind to pathogens. Despite mammals and fish TLRs present functional similarities, there are distinct features in the signaling mechanisms [20,21]. However, LPS induces mRNA expression of molecules involved in inflammation in both mammals and fish [19].

Poly I:C, commonly used to simulate viral infections, interacts with Toll-like 3 receptor (TLR3), expressed in the membrane of B cells, macrophages and dendritic cells. Poly I:C is structurally similar to double-stranded RNA present in viruses and are "natural stimulants" of TLR3. Poly I:C is a synthetic double stranded RNA. So, LPS and poly I:C are used as common tools for scientific research on fish immune system [22].

The present study aimed to evaluate which molecular processes were affected by the exposure of head kidney and liver cells to a surplus concentration of arginine during inflammation. LPS and poly I:C were used to induce cell inflammation and the transcription of selected pro-inflammatory genes (*CD83*, *Cox2*, *IL-1β*, *IL-8*, *INFγ*, *TNFα*, *Mx*, *TLR3* and *viperin*), oxidative genes (*catalase*), apoptotic (*caspase-3*) and anti-apoptotic (*Bcl-2*) genes were monitored in both tissues. Likewise, the transcription of the genes involved in arginine metabolism was also analyzed (*arginase*, *ODC*, *SAMdc* and *SSAT*). Liver cells and head kidney cells isolated from the same individuals, for the comparison of pathogen induced responses in the presence of surplus arginine has to the best of our knowledge, never been published before.

2. Materials and methods

2.1. Fish

Atlantic salmon (*Salmo salar*) were housed by Bergen Aquarium, Norway and fed a commercial diet. The fish from which the cells were isolated had a mean BW of 650 g. For each fish, liver cells and head kidney cells were isolated and cultured in separate wells and plates.

2.2. Culture medium

Complete L-15 medium was prepared as previously described [23]. The L-arginine concentration in this medium is 1817 μM [23].

2.3. Isolation of liver cells

Perfusion and collagenase treatment of salmon liver cells were performed as previously described [23]. The viability of the cells was above 90%.

2.4. Isolation of head kidney cells, modified method

Tissue of head kidney in isolation buffer [23] were aspirated with a syringe and then squeezed through a 40 μM Falcon cell strainer. The cells were washed by centrifugation in a Hettich Zentrifugen, 320R, at 1700 rpm, 5 min, and 4 °C. In this experiment a modified method for the isolation of head kidney cells were applied for the first time: cell pellets were resuspended in the isolation buffer and layered carefully on top of equal amounts of diluted Percoll in density 1.08 g/mL. The tubes were centrifuged at 800 g, 5 min, at room temperature (RT). The cells were collected and pelleted by centrifugation, 1700 rpm 4 °C, for 5 min. An additional washing step was performed before resuspending the cells in cL-15. The viability of the head kidney cells were above 85%.

2.5. Reagents lipopolysaccharide

Reagents lipopolysaccharide was from *Pseudomonas aeruginosa* (LPS, cat# L-7018), Polyinosinic acid: polycytidylic acid, (poly I:C, cat# P9582) and L-arginine (A8094) were purchased from Sigma Aldrich.

2.6. Laminin coating of cell culture wells for liver cell culturing

Wells of 6 well culture plates were coated with laminin (1–2 μg/cm^2, Sigma L2020) for 24 h in room temperature. The laminin solution was then removed and the wells were allowed to dry for 1 h at room temperature before adding the liver cell suspensions.

2.7. Cell cultures

Approximately 1×10^7 of the leukocytes or 0.85×10^6/cm^2 of liver cells isolated from the same fish were added to separate 6 well culture plates (Costar, cat#3335) and cL-15 medium was added to a final volume of 2 ml. The cells were plated in either standard cL-15 medium (measured concentration of 1.82 mM arginine, control) or L-15 medium supplemented with L-arginine to concentrations of 3.63 mM on the day of cell isolation. The cultures were incubated for 2 days in the dark in a normal atmosphere incubator (Sanyo Incubator) at 9 °C. The second day of culturing some of the wells received 100 μg/mL LPS or 50 μg/mL poly I:C. Untreated cultures were included as controls. All cultures were incubated for 24 h post LPS or poly I:C addition before being sampled for analysis.

2.8. Harvesting of cells

The head kidney cells were harvested by centrifugation at day 3. The pelleted cells were homogenized in 600 μl RTL-Plus buffer (RNeasy ®Plus kit Qiagen) using a syringe and frozen at −80 °C before RNA extraction. As the liver cells grow as a monolayer attached to the laminin, these cells were added 600 μl RTL-Plus buffer directly into the cell layer after removing the cell culture supernatant. The harvested cells were frozen at −80 °C before RNA extraction. Identical cell cultures were sampled for protein detection. These cells were washed in PBS and pelleted by centrifugation before dissolving the pellets in 300-μl CelLytic cell lysis reagent (Sigma C2978) according to the manufacturer's instruction. The lysed cells were centrifuged for 15 min at 15 000 g to pellet the cellular debris. The lysates were collected and frozen at −80 °C for further processing using Western Blot.

2.9. RNA extraction

Total RNA was extracted using RNeasy ®Plus kit (Qiagen) according to the manufacturer's instructions, and frozen at −80 °C. The quantity and quality of RNA was assessed using the NanoDrop ND-1000 UV Spectrophotometer (NanoDropTechnologies, Wilmington, DE, USA) and the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). RNA integrity was assessed using the RNA 6000 Nano LabChip® kit (Agilent Technologies, Palo Alto, CA, USA) following the instructions from the supplier. The samples used in this experiment had 260/280 nm absorbance ratios of 2.0 ± 0.1 and 260/230 nm ratios of 2.4 ± 0.1 (mean ± STDEV, n = 24) and RIN-values between 7.5 and 10 indicating RNA samples suitable for RT-qPCR.

2.10. Quantitative real time RT – PCR (qPCR)

The PCR primer sequences, GenBank accession numbers, PCR

Table 1
PCR primer and GenBank accession numbers.

Gene	Forward primer (5' – 3')	Reverse primer (5' – 3')	Accession no.
Arginase	CATAAACACGCCCCTCACCT	GCTTCATCCAGCTGAACCCT	XM_014158368
Bcl-2	TGACAGATTTCATCTACGAGCGG	GCCATCCAGCTCATCTCCAATC	NM_001141086
Caspase 3	ACAGCAAAGAGCTAGAGGTCCAACAC	AAAGCCAGGAGACTTTGACGCAG	DQ008070
Catalase	CCAGATGTGGGCCGCTAACAA	TCTGGCGCTCCTCCTCATTC	Est04a09
CD83	CAAACTGGTCCAGACAGGGT	CAGCGTGATAGACTCGTTC	DQ339141
Cox 2	GGAGGCCTACTCCAACCTATT	CGAACATGAGATTGGAACC	AY848944
IL-1β	GTATCCCATCACCCCATCAC	GCAAGAAGTTGAGCAGGC	NM001123582
IL - 8	GAGCGGTCAGGAGATTTGTC	TTGGCCAGCATCTTCTCAAT	NM_001140710
INF γ	AAGGCGGTCTCGTTAAGT	GCGGCATTACTCCATCCTAA	AJ84811
Mx	TGCCATGCAACGTTGACATTG	GCCTAATGTCCTTTCCCCTTCAG	NM_001139918
ODC	CAGACTGGCAACTCTGCAGC	GCAGCCAAGGCGGTTCACCA	BT045263.1
SAMdc	CTGTCCAGCCTTGCCCCGTG	GGGCCCTGGACTAGTGGGCA	NM_001165344.1
SSAT	TCGTGGCGGAAGTCCCCAGT	GCCGATGCCAAACCCCCTGT	NM_00297.02
TLR3	GTTTCATGGTCAATTACAGTAGG	TGGTTAATGAGTGCAATAGTGG	CB499949
TNF-α	GGCGAGCATACCACTCCTCT	TCGGACTCAGCATCACCGTA	AY848945
Viperin	TCCTTGATGTTGGCGTGGAA	GCATGTCAGCTTTGCTCCACA	NM_001140939

Bcl-2, anti-apoptotic gene; catalase, superoxide dismutase; Cox 2, cyclooxygenase 2; IL-1β, interleukin-1 beta; IL-8, interleukin-8; INFγ, interferon gamma; ODC, Ornithine decarboxylase; SAMdc, S-adenosylmethionine decarboxylase; SSAT, spermidine/spermine-(N1)-acetyltransferase; TLR3, Toll like receptor 3; TNF-α, tumour necrosis factor alpha.

efficiencies and amplicon sizes of the assays used are listed in Table 1. Briefly, the reverse transcription reactions were run in duplicates on 96 well reaction plates with the GeneAmp PCR 9700 machine (Applied Biosystems, Foster City, Ca, USA) using TaqMan reverse transcription reagent containing Multiscribe Reverse Transcriptase (50 U/μL) (N808-0234, Applied Biosystems). For efficiency calculations, a standard dilution curve was recorded using four serial dilutions (500 - 63 ng total RNA) in triplicates. 250 ng RNA was added to the reaction for each sample. No template control (ntc) and no amplification control (sample devoid of RT enzyme) (nac) were run for quality assessment. Reverse transcription reaction was performed starting with an incubation step for 10 min at 25 °C, continuing with RT reaction at 48 °C for 60 min by using oligo dTprimers (2.5 μM) in 50 μL total volume, and finally with 5 min at 95 °C and stored at −20 °C. Gene expression was quantified with qPCR on the Lightcycler 480 (Roche Applied Sciences, Basel Switzerland) on the following program; 5 min activation and denaturizing step at 95 °C followed by 45 cycles of 10 s denaturizing step at 95 °C, 20 s annealing step at 60 °C and a 30 s synthesis step at 72 °C, followed by a melt curve analysis and cooling to 4 °C. The geNorm tool was used to determine a normalization factor from the two reference genes and used to calculate mean normalized expression for the target genes.

2.11. Western Blot

The samples were prepared for SDS-gel by mixing equal amounts of sample and Laemmli sample buffer (BioRad, #161–0737) added 2 β mercapthoethanol (BioRad, #161–0710) before heating the samples for 5 min, 95 °C. Samples and MW-marker (BioRad, #161–0375) were loaded into wells of precast 10% SDS-gels (BioRad, #456–1043) using a BioRad MiniProtean Cell according to the manufacturers instruction. Proteins were blotted onto a polyvinilidene difluoride (PVDF) membrane using the Trans-Blot Turbo Transfer System (BioRad). The membranes were washed 3 × 20 mL PBS/0.1% Tween20, shaking, before blocking the membrane with 5% BSA (Bovine Serum Albumin, Sigma Aldrich,A6003) in PBS/0.1% Tween20 buffer for 1 h, RT, shaking. Primary antibody (1:1000) rabbit anti catalase (#D4P7B XP, Cell Signal Technology) and anti rabbit β-actin (#4967, Cell Signal Technology) were added directly into the blocking solution and incubated overnight at 4 °C, gentle shaking. The membrane was washed as described an added HRP linked secondary antibody (anti-rabbit IgG, #7074, Cell Signal Technology) diluted 1:1000 in ECL Advance Blocking Agent (GE Healthcare, PK 1075). Precision protein StrepTactin-HRP (BioRad # 161–0380), 1:10 000 was also added before incubation for 2 h, RT, shaking. The blot was washed 5 × 10 mL with the washing buffer. Amersham ECL Western Blot Analysis System (#170–5702620, Ge Healthcare) and Chemi Chemiluminiscence Image Capture (Syngene, Cambridge) was used to detect proteins. Signal strength of each specific band was calculated using Gene tools from Syngene, file version 4.03.10, Synoptics Ltd. Relative quantities of catalase compared with β-actin were calculated.

2.12. Statistical analysis

Data comparing gene expression responses between culture conditions were subjected to one-way analyze of variance (ANOVA) using the PROC ANOVA procedure of SAS Version 8.12 software (SAS Institute, Inc., Cary, NC, USA). Differences between treatments within culture conditions were determined by the Student Newman-Keuls test at $P < 0.05$. Western Blot results were analyzed by Two Ways ANOVA, with Tukey's multiple comparison test ($\alpha = 0.05$) as indicated. GraphPad Prisms version 7.0 software was used.

3. Results

3.1. Immune gene responses (Cox2, IL-1β, IL-8, CD83, TNFα, viperin, Mx, INFγ and TLR3, towards LPS and poly I:C, comparing liver and head kidney cells

Poly I:C induced *viperin* transcription in liver cells (Fig. 1a, $p = 0.0132$) and head kidney cells (Fig. 1b, $p < 0.0001$), while *Mx* and *TLR3* were significant upregulated in head kidney cells only (Fig. 1d and h, $p = 0.0009$ and $p = 0.0028$, respectively) following poly I:C challenge.

*INF*γ transcription was not upregulated in either liver cells (Fig. 1e, $p = 0.1367$) or head kidney cells (Fig. 1f, $p = 0.7311$) by any of the treatments.

LPS induced *Cox2*, *IL-1β* and *IL-8* transcription (Fig. 2a–f) both in liver and head kidney cells ($p < 0.0001$ and $p = 0.0288$; $p = 0.0004$ and $p = 0.0013$; $p < 0.0001$ and $p < 0.0001$, respectively). *CD83* was upregulated in liver cells (Fig. 2g, $p < 0.0001$) while *TNFα* was significant upregulated in head kidney cells (Fig. 2j, $p = 0.0021$) following LPS challenge.

3.2. Surplus arginine effects on immune gene responses towards LPS and poly I:C comparing liver and head kidney cells

Poly I:C induced *Mx* transcription was reduced by adding surplus

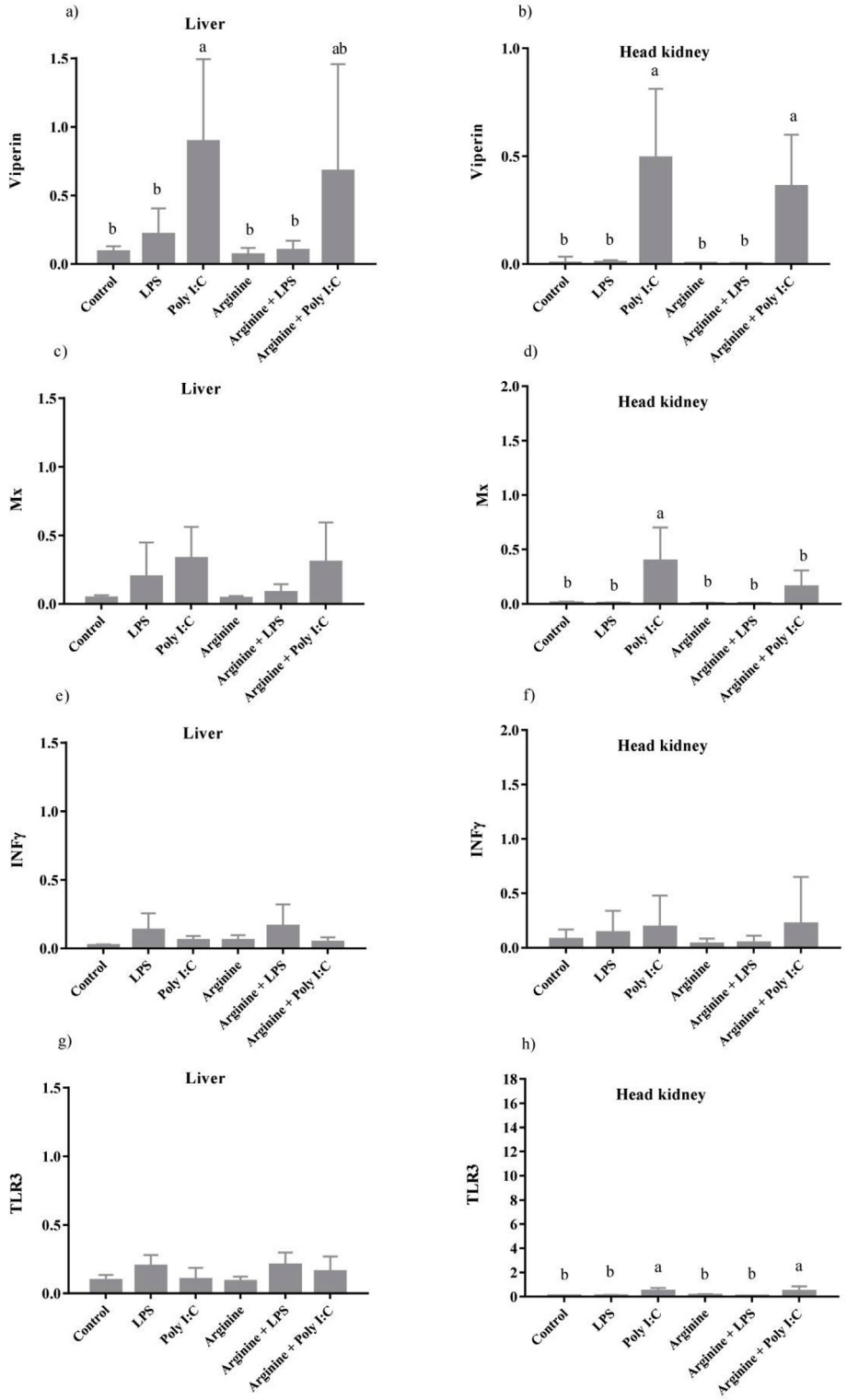

Fig. 1. Inflammation-related genes affected by cell treatment (n = 6). Poly I:C upregulated *viperin* in the liver and head kidney cells (Fig. 1a and b, p = 0.0132 and p < 0.0001, respectively). *Mx* was upregulated by poly I:C in head kidney cells (Fig. 1d, p = 0.0009), but not significantly in liver cells (Fig. 1c). Poly I:C induced *Mx* transcription was downregulated by surplus arginine in head kidney cells (Fig. 1d, p = 0.020). *INF*γ was not affected by any of the cell treatments, both cell types (Fig. 1e and f). Poly I:C upregulated *TLR3* transcription in the head kidney cells (Fig. 1h, p = 0.0028), but not in liver cells (Fig. 1g).

arginine to head kidney cells (Fig. 1d, p = 0.02).

Surplus arginine reduced *Cox2*, *IL-8* and *TNFα* transcription in head kidney cells (Fig. 2b, p < 0.0001, Fig. 2f, p < 0.0001 and Fig. 2j, p = 0.020).

3.3. Gene transcription responses towards LPS and poly I:C related to polyamine synthesis (ODC, SAMdc, Arginase and SSAT), comparing liver and head kidney cells

LPS induced *arginase* transcription in head kidney cells (Fig. 3f,

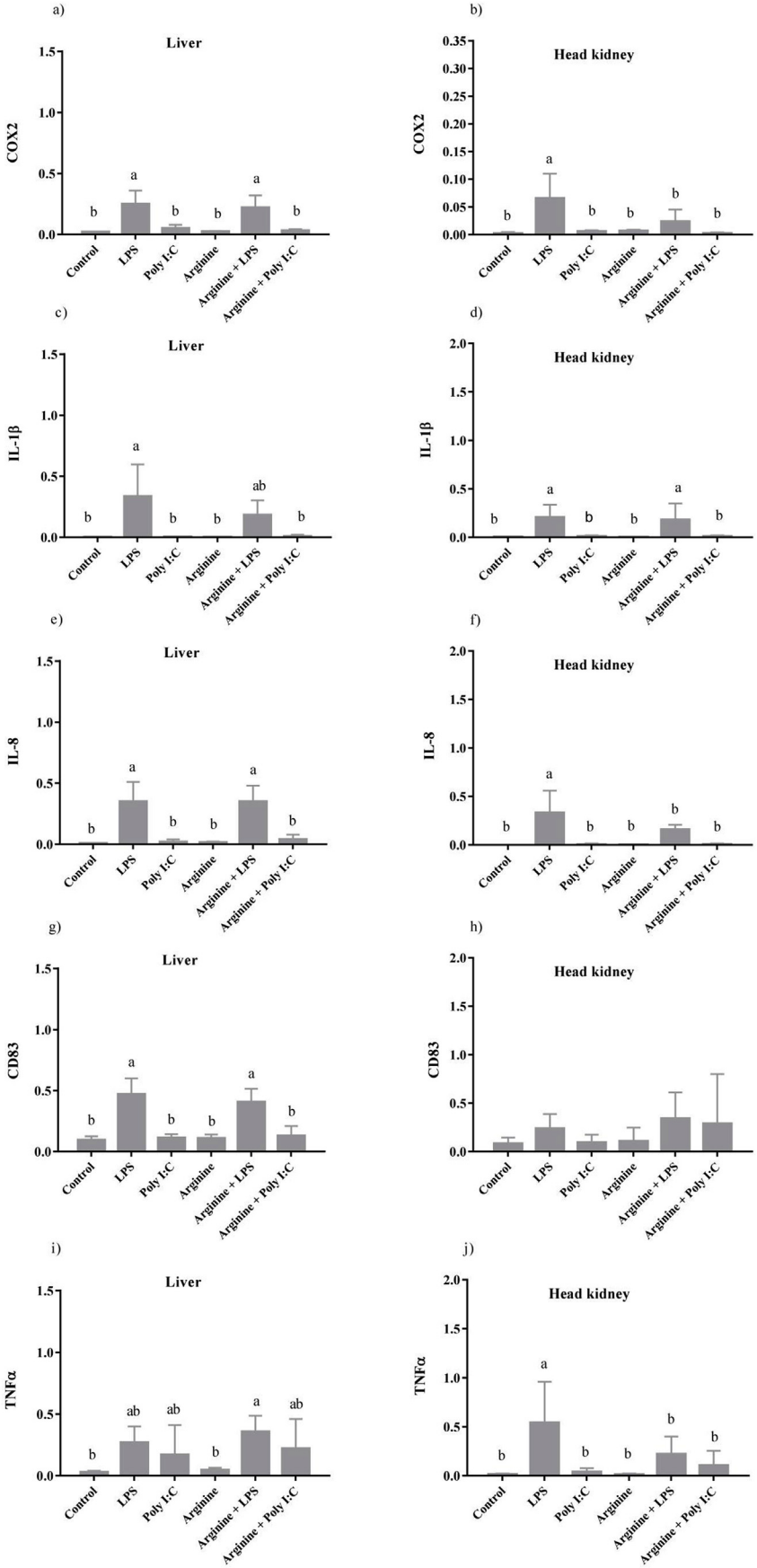

Fig. 2. Inflammation-related genes affected by cell treatment (n = 6). LPS induced *Cox2* transcription in liver and head kidney cells culture (Fig. 2a and b, $p < 0.0001$ and $p = 0.0288$, respectively) and surplus arginine reduced LPS induced *Cox2* transcription in head kidney cells (Fig. 2b, $p < 0.0001$). LPS also induced *IL-1β* (Fig. 2c and d) and *IL-8* (Fig. 2e and f) expression in liver ($p = 0.0004$; $p < 0.0001$) and head kidney cells ($p = 0.0013$; $p < 0.0001$), respectively. Surplus arginine reduced *IL-8* transcription in head kidney cells (Fig. 2f, $p < 0.0001$). *CD83* was upregulated by LPS in the liver cell culture (Fig. 2g, $p < 0.0001$) but not significantly in head kidney cells (Fig. 2h). LPS induced *TNFα* transcription in head kidney cell cultures (Fig. 2j, $p = 0.0021$) but not in liver cells (Fig. 2g). Surplus arginine seemed to be reduce LPS induced expression of *TNFα* transcription in head kidney cell cultures (Fig. 2j, $p = 0.020$).

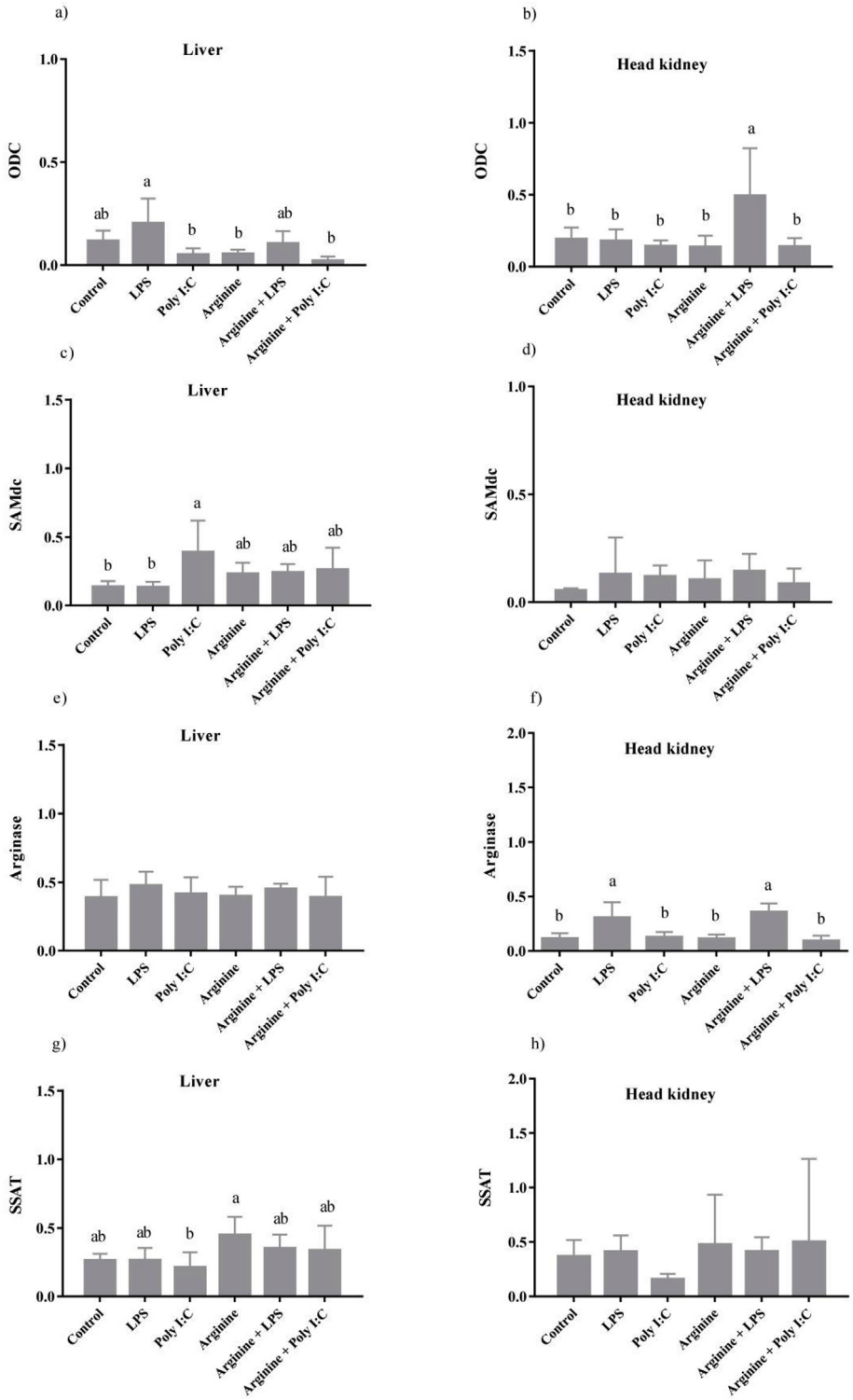

Fig. 3. Genes in polyamine metabolism as affected by cell treatment (n = 6). LPS induced *ODC* transcription when added surplus arginine in head kidney cell culture (Fig. 3b, p = 0.0105), but not in liver cells compared to control (Fig. 3a). Liver cells expressed *SAMdc* induced by poly I:C (Fig. 3c, p = 0.045). LPS induced *arginase* transcription in head kidney cells (Fig. 3f, p < 0.0001), but not in liver cells (Fig. 3e). *SSAT* was not significantly induced in liver cells (Fig. 3g) or in head kidney (Fig. 3h).

p < 0.0001). However, in the liver cell culture, *SAMdc* transcription was significantly upregulated by poly I:C (Fig. 3c, p = 0.045). *SSAT* was not induced either in liver cells (Fig. 3 g, p > 0.05) or in head kidney cells (Fig. 3h, p = 0.7510) by LPS or poly I:C. *ODC* transcription was not affected by LPS and poly I:C alone in liver cells (Fig. 3a) or head kidney cells (Fig. 3b).

3.4. Surplus arginine effects on LPS and poly I:C induced genes related to polyamine synthesis, comparing liver and head kidney cells

ODC transcription was significantly upregulated when LPS and surplus arginine was added together to the head kidney cells (Fig. 3b, p = 0.0105), but not in liver cells compared to control (Fig. 3a).

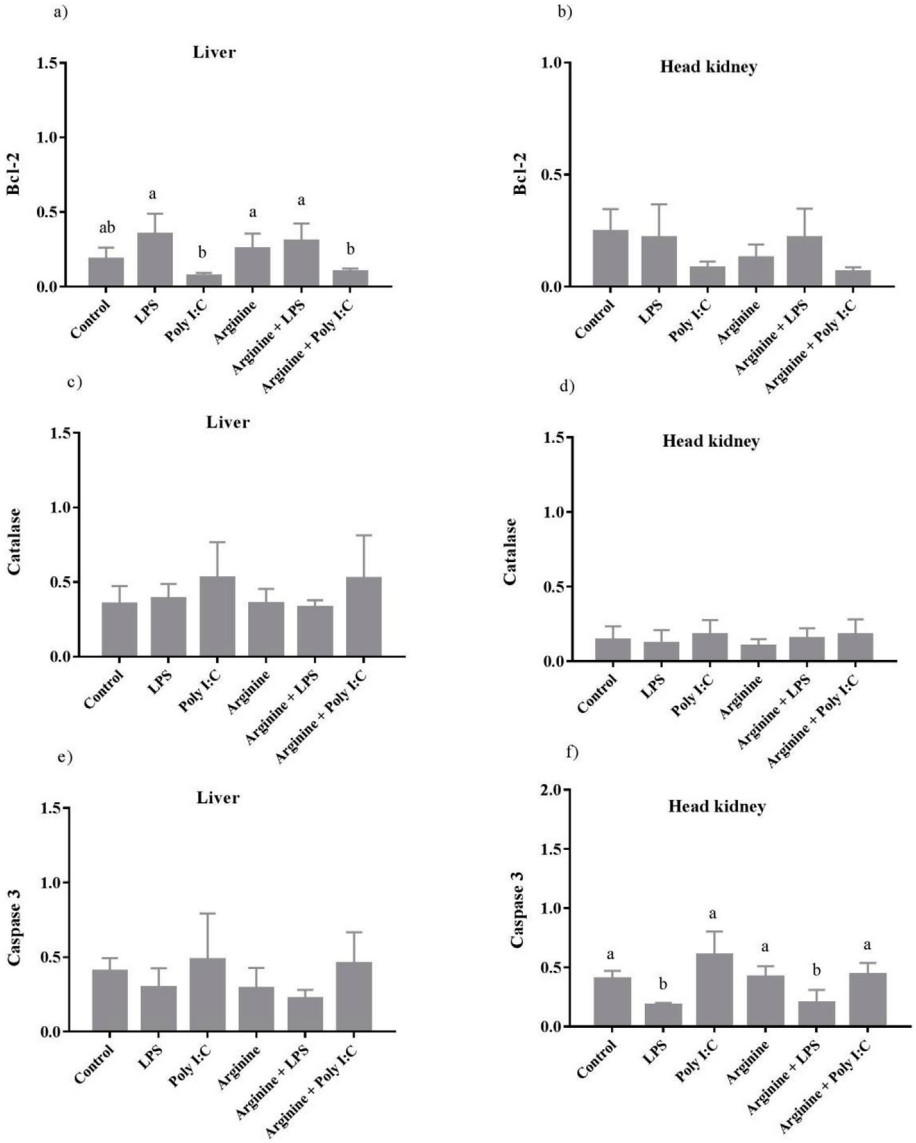

Fig. 4. Apoptosis and antioxidant enzyme related genes affected by cell treatment (n = 6). Poly I:C had no effect on the transcription of *Bcl-2 and catalase* in liver cells (Fig. 4a, c) and head kidney cells (Fig. 4b, d). LPS downregulated *caspase-3* transcription in head kidney cells (Fig. 4f, $p < 0.0001$), but not in liver cells (Fig. 4e).

3.5. Transcription of selected genes induced by LPS and poly I:C related to apoptosis (Bcl-2, caspase-3), antioxidant enzyme (catalase), comparing liver and head kidney cells

In head kidney cells LPS downregulated *caspase-3* transcription (Fig. 4f, $p < 0.0001$), but this was not observed in liver cell cultures (Fig. 4e, $p = 0.1853$). Transcription of *Bcl-2* and *catalase* were not affected by any of the treatments in liver cells or head kidney cells (Fig. 4a, b, $p > 0.05$; 4c, $p = 0.3253$; 4d, $p = 0.7715$) when compared to respective controls.

3.6. Surplus arginine effects of genes induced by LPS and poly I:C related to apoptosis and antioxidant enzyme, comparing liver and head kidney cells

Surplus arginine had no effect on transcription of genes related to apoptosis and antioxidant enzyme in any of the treatments in both cell types (Fig. 4a–f).

3.7. Western Blot results

Surplus arginine, cultured in the presence or absence of LPS, inhibited catalase enzyme production in liver samples, compared to control and LPS (Fig. 5b and c, $p < 0.0001$). Catalase enzyme was not detected in the head kidney cell samples (Fig. 5a). Poly I:C challenge also reduced catalase enzyme expression compared to control in liver cells (Fig. 5b and c, $p < 0.0001$) and surplus arginine significantly increased catalase enzyme expression compared to poly I:C alone.

4. Discussion

An understanding of genes and pathways affected by different stimuli are essential for studying interaction between pathogens and nutrition in farmed salmon. The gene transcription responses to LPS and poly I:C challenge in liver cells and head kidney cells isolated from the same individuals have been elucidated in the current study. In addition, the influence of surplus arginine, an indispensable amino acid for fish [1], when challenged with pathogens, was examined in these *in vitro* models of metabolic cells and immune cells. The results showed that LPS and poly I:C induced quite different transcriptional gene responses in salmon head kidney cells, in line with previously observed responses in Atlantic cod head kidney cells [19]. These responses were quite similar also in salmon liver cells, a pattern that to the best of our

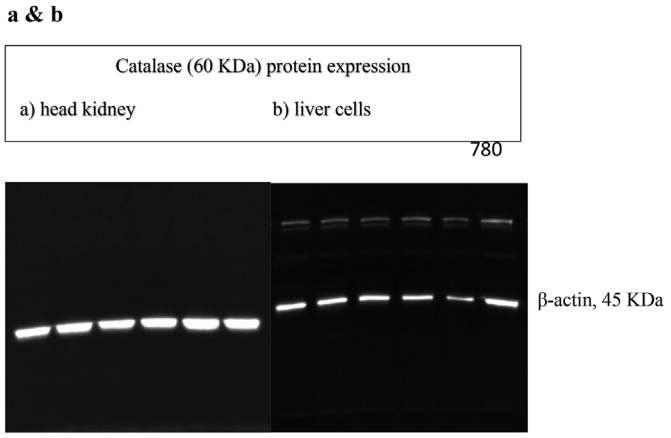

Fig. 5. a & b). Western Blot detecting catalase (60 KDa) protein in head kidney cell lysates (Fig. 5a, n = 3), and liver cell lysates (Fig. 5b, n = 3). No measurable catalase protein was detected in samples from head kidney cells, only β-actin (45 kDa). Catalase was detected in liver cell samples together with β-actin (Fig. 5b).c. Relative expression of catalase protein in liver cell samples (n = 3) when normalized against β-actin. Arginine reduced LPS induced catalase protein. Arginine itself and poly I:C decreased catalase protein expression ($p < 0.0001$) in liver cell samples, compared to control.

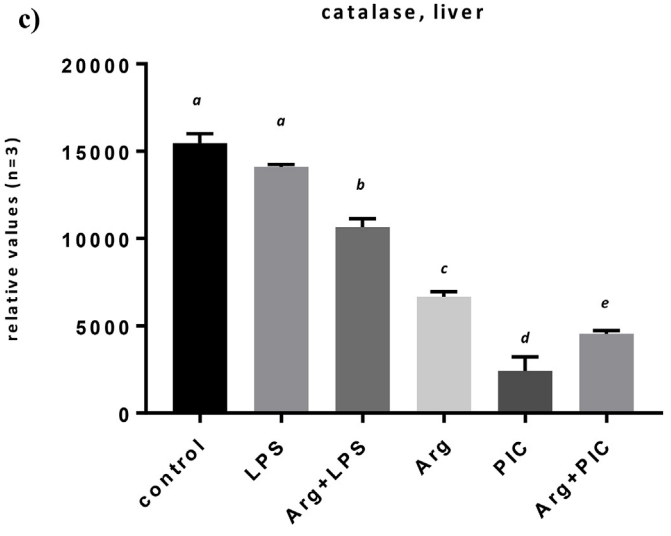

Fig. 5. (*continued*)

knowledge, never have been published before.

The results showed that surplus arginine added to head kidney cells of salmon reduced inflammation by reducing transcription of selected poly I:C and LPS induced proinflammatory genes. IL-1β, IL-8 and TNFα are key mediators of the inflammatory response [24–26] and the expression of such genes are induced by LPS [27,28]. Cox2 is also important in the stages of inflammation mediating the prostaglandin (PG) synthesis [29]. In mammals, the role of PG in inflammatory response included vasodilatation and vascular permeability increase due to the interaction of histamine and bradykinin and the decrease of regulation of leukocytes by the reduction of respiratory burst, proliferation of lymphocytes and antibody production [30].

We observed a strong induction of *IL-1β*, *IL-8* and *Cox2* in salmon liver and head kidney cells stimulated with LPS in line with previous results [21,23]. Surplus arginine inhibited transcription of these genes. This is in contrast to a previous publication [23] adding similar arginine concentration to salmon head kidney cells where no significant immune modulating effects of arginine was observed. The reason for these contradicting results may be that different salmon stocks with different nutritional status were used. However new studies are necessary to evaluate this hypothesis.

CD83 is a marker for dendritic cells (DCs) which are specialized antigen-presenting cells and essential mediators in shaping immune reactivity and tolerance [21,31,32]. *CD83* was expressed in liver cells, but not significantly in head kidney cells when exposed to LPS. However, previous studies reveal that both kinds of cells were involved in the anti-inflammatory responses by regulating the transcription of this gene [21,23].

For several species of fish, sequencing and functional analysis of TLR3 demonstrated the preservation of signaling pathways of TLR3, the involvement in the antiviral immunity and the connection of poly I:C [33–36]. Poly I:C induce antiviral responses in several fish species like Atlantic salmon (*Salmo salar*) [37], common carp (*Cyprinus carpio*) [38] and *Nothobranchius guentheri* [39]. In the present study, poly I:C induced specific antiviral responses (*TLR3, viperin, Mx*) in liver and head kidney cells, suggesting that not only head kidney cells but also liver cells responds to poly I:C. Surplus arginine decreased *Mx* expression in the head kidney cells of salmon, suggesting the ability of arginine to modulate viral responses as well.

Furthermore, in head kidney cells, surplus arginine increased *ODC* transcription when present together with LPS. LPS also induced *arginase* transcription in the heady kidney cells. Arginase converts arginine to urea and ornithine while ODC converts ornithine into putrescine and is considered the rate limiting enzyme for polyamine synthesis [40,41]. These results suggest that LPS interferes with polyamine synthesis in head kidney cells of salmon increasing the transcription rate of enzymes involved in polyamine synthesis. Surplus arginine thus probably is beneficial during bacterial infections. These observations are in line with previous studies indicating that arginine supplementation increased PA synthesis and turnover [10,42]. In the present study, the liver cells altered the *SAMdc* expression following poly I:C challenge. In mammals and fish, SAMdc converts S-adenosyl methionine to polypropyl group necessary for the synthesis of spermidine and spermine [12] suggesting that also poly I:C has the ability to interfere with the polyamine synthesis, at least in liver cells. However, surplus arginine did not have any effects on the pathogen-induced transcription of *arginase*, *SSAT* and *SAMdc* in any of the cell cultures. This is in line with studies on arginine supplementation above the requirements of different species and in cell cultures of salmon hepatic cell culture [5,7,10,41,43]. *SSAT* expression has been reported to be increased by toxins, hormones, cytokines and stress [10,42]. Overexpression of *SSAT* may lead to a variety of biochemical changes, which may cause pathogenesis, like depletion of acetyl-CoA and ATP from the cells, which may lead to formation of reactive species of oxygen, ROS [41,42,44–46].

Apoptosis is an essential process in the development, physiology and homeostasis in the tissues. In mammals, Bcl-2 can inhibit apoptosis dependent on caspases and contribute to tumour cell expansion [47,48]. LPS caused a significant reduction of *caspase-3* in the head kidney cells, in line with Holen and co-workers who reported that LPS induced transcriptional downregulation of several detoxification, apoptotic and antioxidant enzymes in head kidney cells isolated from Atlantic cod and Atlantic salmon [49,50]. Surplus arginine modulated catalase enzyme expression depending on the input signal in liver cell samples. Although gene transcription studies give valuable information, further studies should be carried out to detect arginine effects on enzymes and proteins involved in these pathways.

5. Conclusions

LPS and poly I:C signals through different pathways and affect distinct genes comparing salmon head kidney cells and liver cells isolated from the same fish. Transcription of genes involved in inflammation, polyamine synthesis and redox enzymes were all affected. Surplus arginine reduced transcription of selected LPS and poly I:C induced immune genes in head kidney cells, suggesting that surplus

arginine modulates inflammation. Furthermore, surplus arginine could be beneficial during bacterial challenge, as LPS increased transcription of the rate-limiting enzyme for polyamine synthesis when surplus arginine was present in the head kidney cell cultures. *SAMdc* transcription was upregulated by poly I:C in liver cells, indicating that also poly I:C affects polyamine synthesis.

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