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ORIGINAL PAPER

Effect of water-immersion restraint stress on tryptophan catabolism through the kynurenine pathway in rat tissues

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Abstract The aim of this study was to clarify the effect of water-immersion restraint stress (WIRS) on tryptophan (Trp) catabolism through the kynurenine (Kyn) pathway in rat tissues. The tissues of rats subjected to 6 h of WIRS (+WIRS) had increased tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO) activities and increased TDO and IDO1 (one of two IDO isozymes in mammals) mRNA expression levels, with decreased Trp and increased Kyn contents in the liver. +WIRS rats had unchanged TDO and IDO activities in the kidney, decreased TDO activity and unchanged IDO activity in the brain, and unchanged IDO activity in the lung and spleen, with increased Kyn content in all of these tissues. Pre-treatment of stressed rats with RU486, a glucocorticoid antagonist, attenuated the increased TOD activity, but not the increased IDO activity, with partial recoveries of the decreased Trp and increased Kyn contents in the liver. These results indicate that WIRS enhances hepatic Trp catabolism by inducing both IDO1 and TDO in rats.

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Introduction

In mammals, the catabolism of tryptophan (Trp), an essential amino acid, primarily occurs through the kynurenine (Kyn) pathway. Tryptophan 2,3-dioxygenase (TDO) and indoleamine 2,3-dioxygenase (IDO), two oxidoreductases, catalyze the dioxygenation of L-Trp in the first and rate-limiting step of Trp catabolism. The Kyn pathway ultimately leads to the formation of nicotinamide adenosine dinucleotide from Trp via a series of enzymatic reactions [1]. It is widely accepted that TDO participates in Trp catabolism in liver tissue, while IDO participates in Trp catabolism in extrahepatic tissues. However, several studies have demonstrated the presence of IDO in the livers of rats and mice based on measurements of its enzymatic activity [2–4] and the expression of IDO mRNA [5], respectively, while the presence of TDO in the brain and kidney of rats has also been demonstrated based on measurements of its enzymatic activity [2], and the expression of TDO mRNA in brain tissues in rats and mice [6–8].

There have been multiple reports of a single exposure of rats to several types of stress, such as immobilization stress, cold stress, stress induced by forced running, and stress induced by the Noble–Collip drum procedure, enhancing TDO activity in the liver via the release of corticosteroid hormones secreted from the adrenal gland through the hypothalamus–pituitary–adrenal axis [9–13]. Németh [12] reported that rats exposed to stress induced

by the Noble–Collip drum procedure had a decreased plasma Trp concentration with increased liver TDO activity, although there was no significant change in liver Trp content. A single administration of a high-dose glucocorticoid hormone, such as hydrocortisone or dexamethasone, is well known to induce TDO via transcription of the gene coding for TDO in the liver [14–16]. It is also known that IDO1, one of the two IDO isozymes in mammals, is induced in various extrahepatic tissues and various cell types by pro-inflammatory cytokines such as interferon alpha (INF-α), INF-γ, tumor necrosis factor alpha (TNF-α), and interleukin (IL)-1, especially INF-γ [17–20]. Kiank et al. [21] reported that acute psychological stress (combined acoustic and restraint stress) induces the expression of IDO1 mRNA in the brain, lung, and spleen of mice, leading to an enhancement of IDO1-dependent Trp catabolism in these tissues. These authors have also shown that the induction of IDO1 mRNA expression in the brain of mice with acute psychological stress is mediated by cytokines (e.g., IFN-α, IFN-γ, and TNF-α) [21].

Exposure of experimental animals such as rats and mice to water-immersion restraint stress (WIRS) causes both psychological stress and physical stress. This stress model is widely used in studies aimed at examining the mechanism for stress-induced gastric ulcer in humans because the reproducibility of gastric mucosal lesions is very high in this stress model. The levels of pro-inflammatory cytokines, such as TNF-α and IL-1β, have been found to increase in the plasma and gastric mucosa of rats subjected to WIRS [22–25]. Trp catabolism occurs not only through the Kyn pathway but also through the serotonin (5-hydroxytryptamine) pathway, mainly in extrahepatic tissues such as the brain and gastrointestinal track. The initiating enzyme in the serotonin pathway is tryptophan 5-hydroxylase, and Trp is metabolized in sequential enzymatic steps to 5-hydroxytryptophan, serotonin, and then melatonin through the serotonin pathway; 5-hydroxyindoleacetic acid is also produced as a metabolite of serotonin. Takada et al. [26] showed in rats exposed to WIRS that a decrease in the plasma level of Trp occurs concomitant with increases in the brain levels of serotonin and its metabolite 5-hydroxyindoleacetic acid, although there is no change in the brain level of Trp. However, at the present time it is unclear whether WIRS actually affects Trp catabolism through the Kyn pathway via TDO and/or IDO in the tissues of rats and, if it does play a role in modifying Trp catabolism, how does it do so.

We have therefore examined the effect of WIRS on Trp catabolism through the Kyn pathway in the liver, brain, kidney, lung, and spleen of rats.

Materials and methods

Materials

Hematin, the prosthetic group of heme in TDO, and RU486 (mifepristone), a non-selective glucocorticoid receptor antagonist, were obtained from Sigma-Aldrich Japan (Tokyo, Japan). L-Trp, L-Kyn, corticosterone, ascorbic acid, Tween 80, catalase, and other chemicals were obtained from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). All chemicals used were reagent grade and were not further purified prior to use.

Animals

Male Wistar rats aged 6 weeks were purchased from Nippon SLC Co. (Hamamatsu, Japan). The animals were housed in cages in a ventilated animal room maintained at a controlled temperature (23 ± 2 °C) and relative humidity (55 ± 5 %) under a 12/12-h light/dark (0700–1900/1900–0700 hours) photoperiod. All animals had free access to rat chow (Oriental MF; Oriental Yeast Co., Tokyo, Japan) and tap water for 1 week prior to the experiments. All animals received humane care in compliance with the Guidelines of the Management of Laboratory Animals in Fujita Health University which are based on Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of the Ministry of Education, Culture, Sports, Science and Technology in Japan (Notice No. 71, 2006). The animal experiment was approved by the Institutional Animal, Care and Use Committee (Protocol nr. M1461).

Induction of WIRS

At the time of the experiments all rats were 7 weeks old and had been starved for 24 h before the start of the WIRS treatment but allowed free access to water. The rats were first weighed and then divided into two groups, i.e., one group to be exposed to WIRS (+WIRS) and the control group which was not exposed to WIRS (−WIRS). All rats in the +WIRS group were restrained in wire cages and immersed up to the depth of the xiphoid process in a 23 °C water bath to induce WIRS, as described in our previous reports [27–30]. Exposure of rats to WIRS was started at 0900 hours and maintained for 6 h. The corresponding control rats (−WIRS) were maintained at room temperature for the same period of time. The duration of WIRS exposure chosen in this study was based on the results of our previous studies [27–30] which showed that exposure to 6 h of WIRS causes apparent stress responses in rats,

based on increased levels of serum adrenocorticotrophic hormone, corticosterone, and glucose levels and the formation of gastric mucosal lesions.

RU486 treatment

RU486 was dissolved in 5 % Tween 80 (200 mg/mL) and injected intraperitoneally into rats at a dose of 20 mg/kg body weight at 30 min before the onset of WIRS. Rats without RU486 treatment were injected intraperitoneally with the same volume of 5 % Tween 80 at the same time point. The dose of RU486 used was determined based on a previous study [31] showing that impairment of hippocampal/prefrontal long-term potentiation, one of main stress-induced features, was effectively suppressed by a single intraperitoneal injection of RU486 at a dose of 20 mg/kg body weight before the onset of stress exposure.

Sample preparation

Immediately after the end of the 6-h exposure to WIRS, the rats were weighed and then sacrificed under pentobarbital anesthesia at which time blood was collected from the inferior vena cava. Serum was separated from the collected blood by centrifugation. Immediately after death, the portal vein was perfused with ice-cold 0.9 % NaCl to remove any blood remaining in the tissues, and then the livers, kidneys, brains, lungs, and spleens were removed. The removed tissues were washed with ice-cold 0.9 % NaCl, patted dry on filter paper, and then weighed. The tissues and serum thus obtained were stored at −80 °C until use.

Assays of serum corticosterone, glucose, and cytokines

Serum corticosterone was fluorometrically assayed using the method of Guillemin et al. [32] with authentic corticosterone as a standard. Serum glucose was determined using the Glucose CII-Test kit (Wako Pure Chemical Ind., Ltd.). Serum INF-γ was assayed using the Cytometric Bead Array Rat INF-γ Flex set (Becton, Dickinson and Company, Franklin Lake, NJ). Liver INF-γ was assayed using the Rat INF-gamma Quantikine ELISA kit (R&D Systems, Inc., Minneapolis, MN). TNF-α and IL-1β in both serum and liver tissues were assayed using the Rat TNF-alpha Quantikine ELISA kit and Rat IL-1 beta Quantikine ELISA kit (R&D Systems, Inc.), respectively. The assays of INF-γ, TNF-α, and IL-1β in serum and liver tissues were performed according to the manufacturer's recommendations.

Assays of TDO, IDO, and protein in tissues

Tryptophan 2,3-dioxygenase in liver, kidney, and brain tissues and IDO in liver, brain, kidney, lung, and spleen tissues were assayed as described previously [33]. Briefly, tissues were disrupted with a Polytron homogenizer (Kinematica Lab Equipment CSC, Tokyo, Japan) using 1.5–5 volumes of ice-cold 0.14 M KCl–20 mM potassium phosphate buffer (pH 7.0). The homogenates were centrifuged at 4 °C (14,000 *g*, 10 min), and the resulting supernatants were used for the TDO and IDO assays. The reaction mixture for each enzyme assay consisted of 50 μL of enzyme preparation and 50 μL of substrate solution. The composition of the substrate solution for the TDO assay was 100 mM potassium phosphate buffer (pH 6.5), 50 mM ascorbic acid, 0.5 mg hematin, and 2 mM L-Trp. The composition of the substrate solution for the IDO assay was 100 mM potassium phosphate buffer (pH 6.5), 50 mM methylene blue, 20 mg of catalase, 50 mM ascorbic acid, and 0.4 mM L-Trp. In both the TDO and IDO assays, the reaction mixture was incubated at 37 °C for 60 min. Just before and after incubation, the reaction mixture for each sample was acidified by adding 100 μL of 3 % perchloric acid followed by centrifugation at 4 °C (14,000 *g*, 10 min). The level of Kyn in the reaction mixtures of each sample, both those subjected to the 60-min incubation, or not, was measured by high-performance liquid chromatography (HPLC; see section Measurements of Trp and Kyn in serum and tissues). The amount of Kyn formed enzymatically in each sample was calculated by subtracting the amount of Kyn determined without incubation from that amount determined after the 60-min incubation. The activities of TDO and IDO are expressed as the amount of Kyn formed per hour per milligram protein. Protein in tissue samples was assayed using a commercial Rapid Protein Assay kit (Wako Pure Chemical Ind. Ltd.). Bovine serum albumin was used as a standard in this protein assay.

RNA isolation and reverse transcription-PCR analysis

Total RNA was extracted from liver tissues with ISOGEN (Nippon GENE, Tokyo, Japan), and the RNA concentration in each extract was determined spectrophotometrically at 260 nm. Reverse transcription (RT)-PCR was performed using Revetra Ace kits (TOYOBO Co., Ltd., Osaka, Japan). Primers used in the semi-quantitative analysis were:

β-Actin: 5′-ATGGATGACGATATCGCT-3′ (sense) and 5′-ATGAGGTAGTCTGTAGGT-3′ (antisense)

Rat TDO: 5′-CAGAGCTTGAGACTCCCCTTA-3′ (sense) and 5′-GTCGTCGTTCACCTTTACTC-3′ (antisense)
Rat IDO1: 5′-GCTGCCTCCCATTCTCTCTT-3′ (sense) and 5′-TGCGATTTCCACCATTAGAGAG-3′ (antisense).

Semi-quantitative analysis of RT-PCR products was performed by using NIH ImageJ 1.34 s software and normalized to β-actin. When the same experiment was repeated three times, the three experiments showed similar results. Therefore, the results obtained from only one of the three experiments were analyzed.

Measurements of Trp and Kyn in serum and tissues

Tryptophan and Kyn levels in the serum and tissues were measured by HPLC as reported previously [34]. For the serum measurements, the serum was first mixed with one volume of 10 % (v/v) perchloric acid for deproteinization, followed by incubation of the mixture on ice for 10 min and then centrifugation at 4 °C (7000 *g*, 10 min); Trp and kyn measurements were made on the resultant supernatant. For the Trp and Kyn measurements in the tissues, each tissue was first homogenized in 1.5 volume of 3 % perchloric acid and the homogenate then kept at 4 °C overnight followed by centrifugation at 4 °C (7000 *g*, 20 min). The resultant supernatant was loaded on Ultrafree-MC Centrifugal Filter Units with microporous membrane (pore size: 0.45 μm; Millipore, Tokyo, Japan) and centrifuged at 4 °C (12,000 *g*, 4 min); the supernatant from this last step was analyzed by HPLC (sample size 50 μL). Trp and Kyn were isocratically eluted from a reverse-phase column [TSKgel ODS-100v 3 μm: 4.6 mm (internal diameter) × 15 cm (length); Tosoh Corp., Tokyo, Japan] using a mobile phase containing 10 mM sodium acetate and 1 % acetonitrile (pH adjusted to 4.5 with acetic acid) at a flow rate of 0.9 mL/min. Trp and Kyn were detected by UV-spectrophotometry (model SPD-20A; Shimadzu, Kyoto, Japan) at a UV wavelength of 280 nm for Trp and 365 nm for Kyn. The retention times of Trp and Kyn under these measurement conditions were around 12.5 and 7.4 min, respectively.

Statistical analysis

All data were expressed as the mean ± standard deviation (SD). Statistical analysis was performed using the unpaired Student's *t* test. For values obtained in the experiment with RU486 pretreatment, statistical analysis was performed using one-way analysis of variance followed by the Bonferroni/Dunn test for multi-comparison. The *P* value of <0.05 was considered to be statistically significant.

Results

Serum glucocorticoid and glucose levels, body weight, and tissue weight in +WIRS rats and −WIRS rats

As shown in Fig. 1, serum corticosterone and glucose concentrations, which are indices used to reflect stress responses, were significantly higher in the +WIRS rats than in the −WIRS (control) rats, with serum corticosterone and glucose concentrations in the stressed rats being 2.2- and 2.2-fold higher, respectively, than those in the unstressed rats.

When rats were weighed just before the initiation of the WIRS experiment, the mean body weight of rats in the +WIRS group and −WIRS group was 215 ± 7 g ($n = 8$) and 209 ± 6 g ($n = 7$), respectively. There was no significant difference in body weight between these two groups of rats. When the body weight of the rats was measured after exposure of the +WIRS group to 6 h of WIRS, the body weight of rats in the +WIRS group (212 ± 7 g; $n = 8$) did not differ significantly from that of rats in the −WIRS group (207 ± 4 g; $n = 7$). There was also no significant difference in the body weight of the rats in each group between before and after WIRS exposure. The weights of the livers, brains, kidneys, and lungs of +WIRS rats ($n = 8$) did not significantly differ from those of the −WIRS rats ($n = 7$) as follows: liver, 7.87 ± 0.44 (+WIRS) versus 7.92 ± 1.05 g (−WIRS); brain, 1.99 ± 0.04 (+WIRS) versus 2.02 ± 0.06 g (−WIRS); kidney (a pair of kidneys), 1.85 ± 0.09 (+WIRS) versus 1.92 ± 0.15 g (−WIRS); lung, 1.07 ± 0.11 (+WIRS) versus 1.02 ± 0.08 g (−WIRS). In contrast, the spleens of +WIRS rats were significantly lighter than those of −WIRS rats [0.34 ± 0.04 ($n = 8$) vs. 0.45 ± 0.04 g ($n = 7$), respectively ($P < 0.05$)].

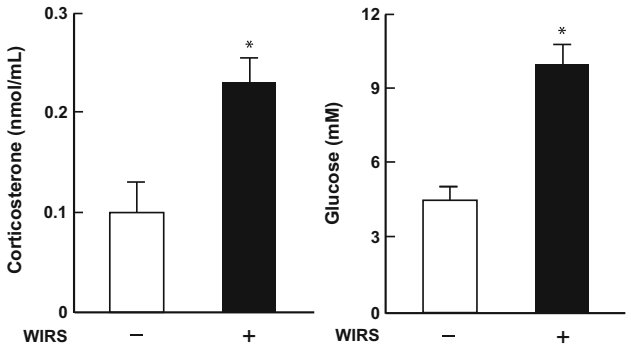

Fig. 1 Serum corticosterone and glucose levels in rats subjected (+) or not (−) to 6 h of water-immersion restraint stress (*WIRS*) based on assays described in the Materials and methods. Each value is the mean ± standard deviation (SD) ($n = 7$ rats in the −WIRS group; $n = 8$ rats in the +WIRS group). *$P < 0.05$ (vs. −WIRs rats)

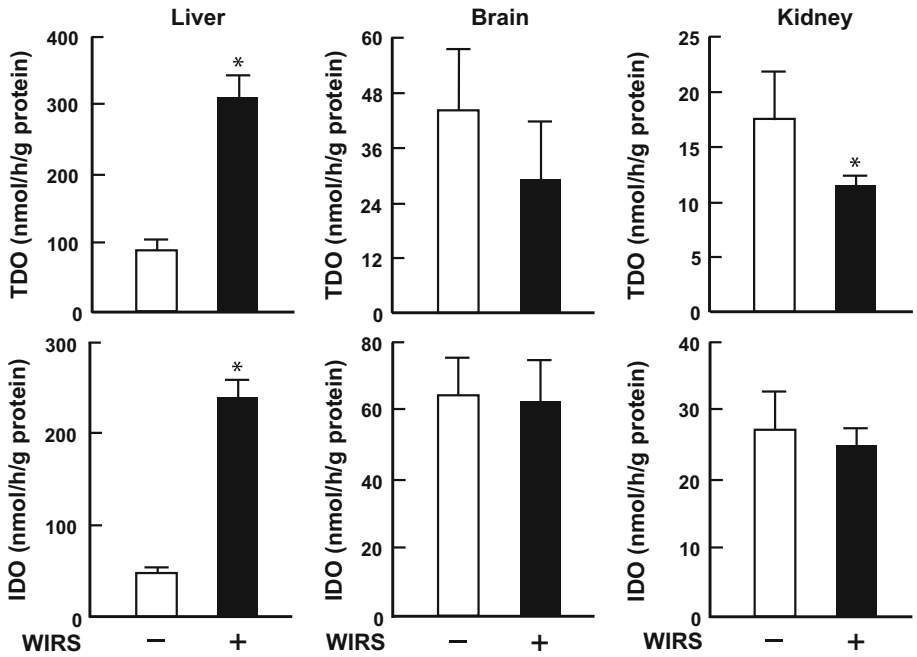

Fig. 2 Tryptophan 2,3-dioxygenase (*TDO*) and indoleamine 2,3-dioxygenase (*IDO*) activities in the liver, brain, and kidney tissues of rats subjected (+) or not (−) to 6 h of WIRS based on assays of TDO and IDO described in the Materials and methods. Each value is the mean ± SD ($n = 7$ −WIRS rats; $n = 8$ +WIRS rats). *$P < 0.05$ (vs. −WIRS rats)

Liver, brain, and kidney TDO and IDO activities and lung and spleen IDO activities in +WIRS rats and −WIRS rats

Liver TDO and IDO activities were significantly higher in +WIRS rats than in −WIRS rats, with TDO and IDO activities in +WIRS rats being 3.6- and 5.2-fold higher, respectively, than those in unstressed rats (Fig. 2). There were no significant differences in TDO and IDO activities in the brain between +WIRS and −WIRS rats, although TDO activity in the brain tended to be lower in the former than in the latter (Fig. 2). TDO activity in the kidney was significantly lower in +WIRS rats than in −WIRS rats, but there was no significant difference in IDO activity in the kidney between +WIRS and −WIRS rats (Fig. 2). IDO activity in the lung of −WIRS rats ($n = 7$) and +WIRS rats ($n = 8$) was 69.2 ± 13.3 and 66.4 ± 14.2 nmol Kyn/h/g protein, respectively. IDO activity in the spleen of −WIRS ($n = 6$) and +WIRS rats ($n = 7$) was 92.1 ± 4.2 and 88.1 ± 9.3 nmol Kyn/h/g protein, respectively. There were no significant differences in IDO activities in the lung and spleen between +WIRS and −WIRS rats ($P > 0.05$).

Levels of TDO and IDO1 mRNA expression in liver tissues of +WIRS and −WIRS rats

As shown in Fig. 3, the levels of liver TDO and IDO1 mRNA expression were significantly higher in +WIRS rats than in −WIRS rats, with the levels of liver TDO and IDO1 mRNA expression in the stressed rats being 2.0- and 1.9-fold higher, respectively, than those in unstressed rats.

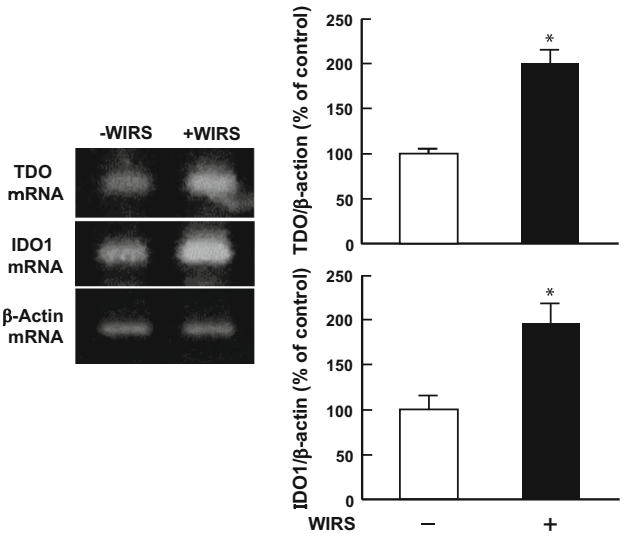

Fig. 3 Levels of TDO and IDO1 mRNA expression in liver tissues of rats subjected (+) or not (−) to 6 h of WIRS based on assays of TDO and IDO1 described in the Materials and methods. Each value is the mean ± SD ($n = 7$ −WIRS rats; $n = 8$ +WIRS rats). *$P < 0.05$ (vs. −WIRS rats)

Trp and Kyn levels in serum and various tissues of +WIRS and −WIRS rats

Serum Trp concentration and liver Trp content were significantly lower in +WIRS rats than in −WIRS rats, with serum Trp concentration and liver Trp content in the stressed rats being 26.9 and 88.6 % of those in the unstressed rats, respectively (Fig. 4). There were no

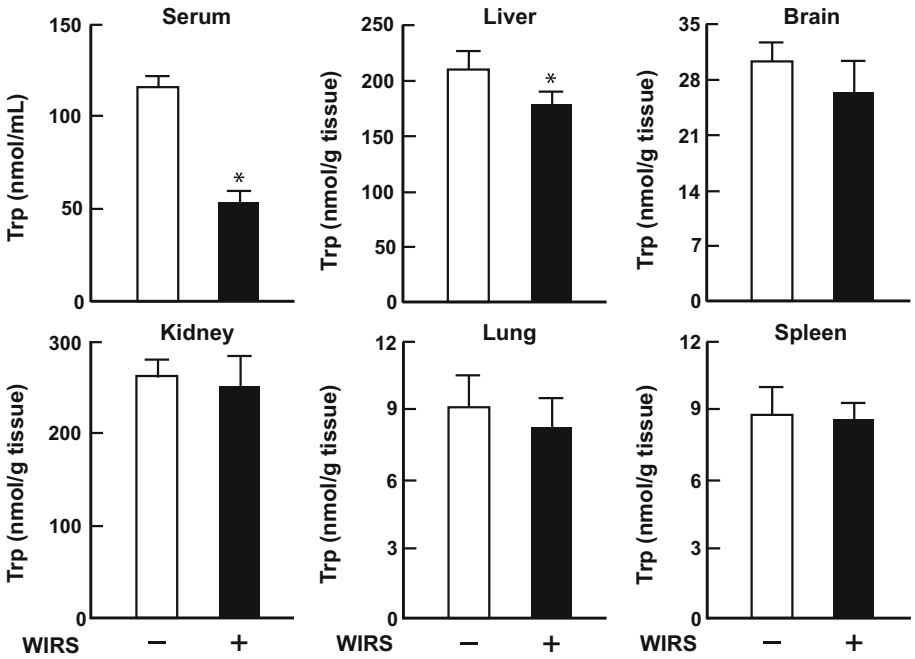

Fig. 4 Concentration of tryptophan (*Trp*) in serum and levels of Trp in various tissues of rats subjected (+) or not (−) to 6 h of WIRS based on assays of Trp described in the Materials and methods. Each value is the mean ± SD ($n = 7$ −WIRS rats; $n = 8$ +WIRS rats). *$P < 0.05$ (vs. −WIRS rats)

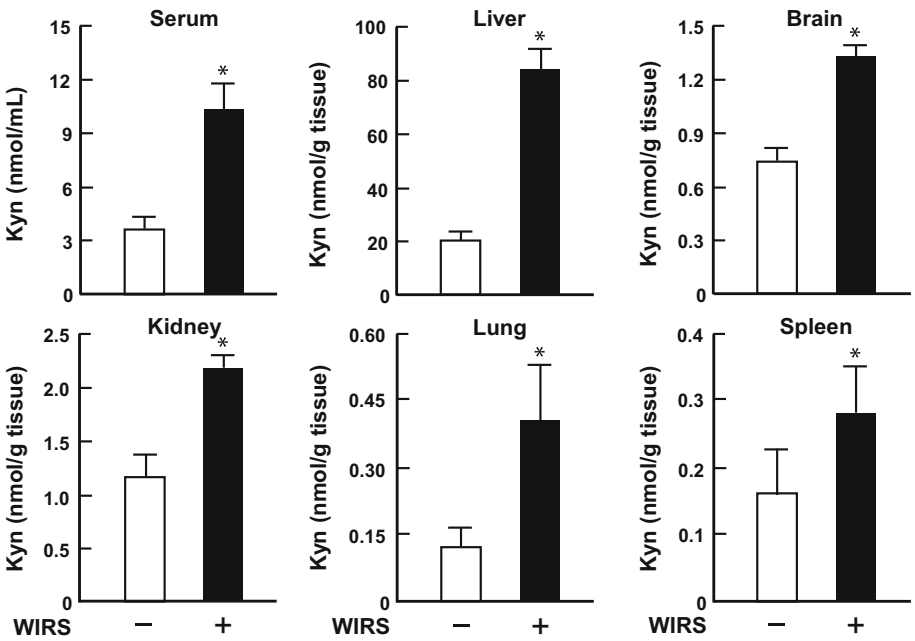

Fig. 5 Concentration of kynurenine (*Kyn*) in serum and levels of Kyn in various tissues of rats subjected (+) or not (−) to 6 h of WIRS based on assays of Kyn described in the Materials and methods. Each value is the mean ± SD ($n = 7$ −WIRS rats; $n = 8$ +WIRS rats). *$P < 0.05$ (vs. −WIRS rats)

significant differences in brain, kidney, lung, and spleen Trp contents between +WIRS and −WIRS rats (Fig. 4). As shown in Fig. 5, the serum Kyn concentration and Kyn contents in liver, brain, kidney, lung, and spleen tissues were significantly higher in +WIRS rats than in −WIRS rats, with the serum Kyn concentration and liver, brain, kidney, lung, and spleen Kyn contents in the stressed rats being 3.5-, 4.1-, 1.8-, 1.9-, 3.3-, and 1.7-fold higher, respectively, than those in the unstressed rats (Fig. 5).

IFN-γ, TNF-α, and IL-1β levels in serum and liver tissues of +WIRS and −WIRS rats

Serum IFN-γ, TNF-α, and IL-1β levels were significantly higher in +WIRS rats than in −WIRS rats (Fig. 6). The level of IFN-γ in the liver of +WIRS rats was significantly higher than that in −WIRS rats, but there were no significant differences in the liver TNF-α and IL-1β levels between +WIRS rats and −WIRS rats (Fig. 6).

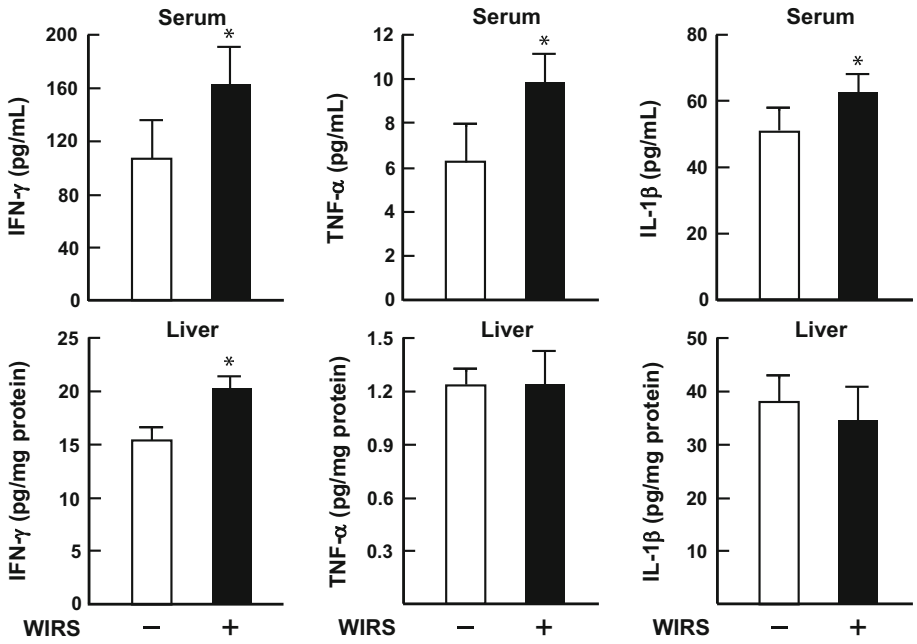

Fig. 6 Interferon gamma (*IFN-γ*), tumor necrosis factor alpha (*TNF-α*), and interleukin 1beta (*IL-1β*) levels in serum and liver tissues of rats subjected (+) or not (−) to 6 h of WIRS based on assays described in the Materials and methods. Each value is the mean ± SD ($n = 7$ −WIRS rats; $n = 8$ +WIRS rats). *$P < 0.05$ (vs. −WIRS rats)

Effect of RU486 pretreatment on changes in serum glucocorticoid, glucose, Trp, and Kyn levels in +WIRS and −WIRS rats

The increase in serum corticosterone concentration found in the stressed (+WIRS) rats was further enhanced significantly by RU486 pretreatment, while the increase in serum glucose concentration found in the +WIRS rats was significantly reduced by the pretreatment (Fig. 7). Both the decrease in serum Trp concentration and the increase in serum Kyn concentration found in +WIRS rats were significantly attenuated by RU486 pretreatment (Fig. 7).

Effect of RU486 pretreatment on changes in liver TDO and IDO activities and Trp, Kyn, and INF-γ levels in +WIRS and −WIRS rats

Pretreatment with RU486 significantly reduced the increases in liver TDO activity and Kyn content and the decrease in liver Trp content found in +WIRS rats, while the pretreatment had no significant effect on the increases in liver IDO activity and INF-γ level found in the stressed rats (Fig. 8).

Discussion

The results of this study show that a single exposure of fasted rats to 6 h of WIRS (combined psychological and physical stress) induced apparent stress responses, based on marked increases in serum corticosterone and glucose

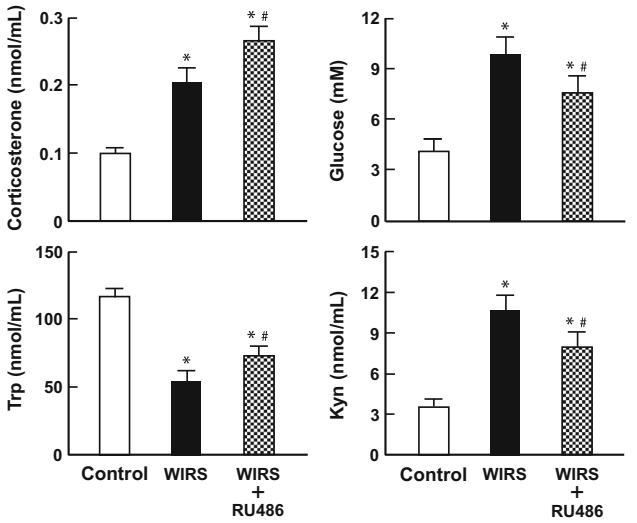

Fig. 7 Effect of RU486 (mifepristone; a non-selective glucocorticoid receptor antagonist) pretreatment on changes in serum corticosterone, glucose, Trp, and Kyn levels in rats subjected (*WIRS*) or not (*Control*) to 6 h of WIRS. Rats in the WIRS group were injected intraperitoneally with either RU486 (*WIRS + RU486*; 20 mg/kg body weight) or vehicle (*WIRS*; 0.5 % Tween 80) at 0.5 h before the onset of the WIRS treatment. Rats in the Control group were injected intraperitoneally with vehicle in the same manner and at the same time point. Serum corticosterone, glucose, Trp, and Kyn levels in each group were determined using the assays described in the Materials and methods. Each value is the mean ± SD ($n = 7$ Control rats; $n = 8$ WIRS rats; $n = 8$ WIRS + RU486 rats). *$P < 0.05$ (vs. Control rats); #$P < 0.05$ (vs. +WIRS rats without RU486 pretreatment)

levels. These findings are in agreement with those reported previously [27–29]. We found no differences in body weight and the weights of the liver, brain, kidney, and lung

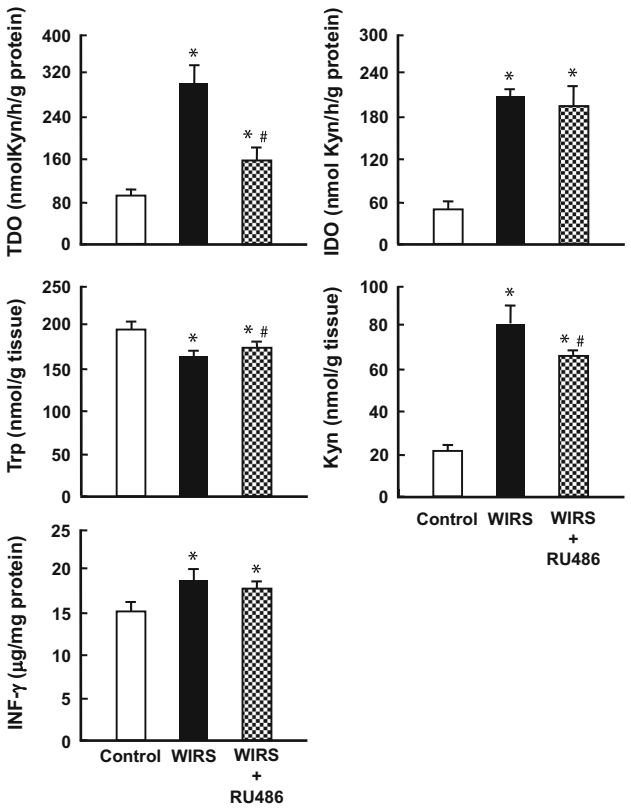

Fig. 8 Effect of RU486 pretreatment on changes in TDO and IDO activities and Trp, Kyn, and INF-γ levels in liver tissues of rats subjected (*WIRS*) or not (*Control*) to 6 h of WIRS treatment. Rats in the WIRS group were injected intraperitoneally with either RU486 (*WIRS + RU486*; 20 mg/kg body weight) or vehicle (*WIRS*; 0.5 % Tween 80) at 0.5 h before the onset of the WIRS treatment. Rats in the Control group were injected intraperitoneally with vehicle in the same manner and at the same time point. Liver TDO and IDO activities and liver Trp, Kyn, and INF-γ levels in each group were determined using the assays described in the Materials and methods. Each value is the mean ± SD ($n = 7$ Control WIRS; $n = 8$ WIRS rats; $n = 8$ WIRS + RU486 rats). $^{*}P < 0.05$ (vs. Control rats); $^{\#}P < 0.05$ (vs. WIRS rats)

between the stressed rats (+WIRS) and non-stressed rats (−WIRS). However, the spleen weighed less in the stressed rats than in the unstressed ones, leading us to assume that in rats exposed to WIRS, the spleen tissue is more sensitive to stress than the liver, brain, kidney, and lung tissues. A single exposure of rats to several types of stress, such as immobilization stress, cold stress, forced running-induced stress, and stress induced by the Noble–Collip drum procedure, is known to induce TDO in liver tissue [9–13]. Both TDO and IDO are present in the liver of rats [2–4], but it has long been thought that TDO is localized in the hepatic tissue of mammals, while IDO is localized in the extrahepatic tissues of mammals. In our rat model system, a single exposure to 6 h of WIRS markedly increased not only TDO activity but also IDO activity in the liver tissue. Although both IDO and TDO are known to be present in the brain and kidney of rats [2], in our study a single exposure to 6 h of WIRS did not affect TDO and IDO activities in the brain of +WIRS rats, while it did reduce TDO activity without affecting IDO activity in the kidney. The single treatment also had no effect on IDO activity in the lung and spleen. Thus, we found that IDO activity was not increased in the brain, lung, and spleen of our +WIRS rats, although it has been previously shown that acute psychological stress (combined acoustic and restraint stress) does induce the expression of IDO1 mRNA in the brain, lung, and spleen of mice, leading to an enhancement of IDO1-dependent Trp catabolism in these tissues [20]. We did observe that a single exposure of rats to WIRS for 6 h caused marked increases in the expression levels of TDO and IDO1 mRNA in the liver. To date, two IDO isoenzymes, i.e., IDO1 and IDO2, have been identified in mammals such as mice, dogs, cows, and humans, although the IDO2 present in mammals has a relative low affinity for Trp [35]. In mice, IDO2 protein is predominantly expressed in the kidney, followed in decreasing order of abundance by the epididymis, testis, and liver [36, 37]. At the present time, however, there is no available information on the presence of IDO2 in the liver tissue and cells of rats. Our results indicate that a single exposure of rats to WIRS causes both IDO1 and TDO induction at the level of mRNA expression and enzymatic activity in liver tissue. As such, this is the first report of IDO1 induction in liver tissues of mammals under stressful conditions. Fracesconi et al. [38] reported that increased urinary excretory output of the two major metabolites of Trp, namely, kynurenic acid and xanthurenic acid, occurs in humans exposed to acute cold stress. This finding led these authors to suggest that the alteration in plasma Trp level in humans exposed to acute cold stress is mediated through an increase in the activity of TDO. Okamoto et al. [39] also reported that increased urinary excretory output of the catabolites of niacin, which is produced from Trp via the Kyn pathway in the liver, occurs in humans exposed to acute cold stress, further suggesting that the biosynthesis of nicotinamide from Trp is increased by exposure to acute cold stress. Accordingly, these findings may allow us to assume that not only TDO induction but also IDO1 induction participates in enhanced Trp catabolism in the liver of humans exposed to cold stress.

TDO and IDO1 participate in Trp catabolism as the first and rate-limiting enzyme in tissues of mammals and metabolize L-Trp to *N*-formylkynurenine, resulting in the formation of L-Kyn via kynurenine formamidase, in the tissues. To verify whether induced TDO and IDO1 enhance Trp catabolism in the liver of rats exposed to 6 h of WIRS, we examined changes in Trp and Kyn levels in the serum, liver, brain, kidney, lung and spleen of the stressed (+WIRS) rats and found that +WIRS rats showed a

marked reduction in serum Trp concentration and a marked increase in serum Kyn concentration. There was a significant reduction in liver Trp content in the +WIRS rats, but the reduced amount of Trp in the liver was equivalent to approximately 10 % of Trp content in the liver of the unstressed (−WIRS) rats. However, the +WIRS rats showed no change in Trp content in the brain, kidney, lung, and spleen. In contrast, we observed a significant increase in Kyn content in the liver, brain, kidney, lung, and spleen of +WIRS rats, with the largest increase in Kyn content being in the liver. These results support the premise that TDO and IDO1 are indeed induced at the level of enzymatic activity in the liver of rats exposed to WIRS. Nevertheless, the reduction in Trp content was not as large as the increase in Kyn content in the liver of +WIRS rats, as described above. Saito et al. examined Trp uptake in hepatocytes isolated from the liver of hydrocortisone-treated rats and reported that Trp uptake into the isolated hepatocytes occurred via a high-affinity transport system that works under physiological conditions and which is stimulated under Trp depletion due to induced TDO [40]. Therefore, one possible explanation for our results is that an active uptake of Trp from the blood stream occurs in the liver of rats exposure to WIRS, leading to compensation of Trp metabolized by the induced TDO and IDO1 in the tissue. Indeed, we found a marked reduction in Trp concentration in the serum of +WIRs rats, as described above. However, the +WIRS rats showed an apparent increase in Kyn content not only in the liver but also in the brain, kidney, lung, and spleen despite the absence of any observable significant change in Trp content in these tissues, as described above. Such an increase in Kyn level without any change in Trp level has been demonstrated previously in the plasma, liver, and kidney of rats exposed to foot shock [41]. Young [42] suggested that the decline in brain Trp content caused by Trp depletion due to a high activity of liver TDO is diminished by the release of Trp from proteins in peripheral tissues in rats treated with hydrocortisone. Accordingly, one can assume that, in rats exposed to WIRS, Trp released from proteins in the peripheral tissues is transported to the brain where it is catabolized to Kyn via TDO and/IDO in the tissue, resulting in the maintenance of the original Trp level despite an increase in Kyn level in the tissue. One possibility is that Trp released from proteins in the kidney of rats with WIRS is metabolized by TDO and/or IDO in the tissue, resulting in an increase in Kyn content without any change in Trp content in the tissue. Furthermore, it may be possible to assume that Trp released from proteins in the lung and spleen of +WIRS rats is metabolized by IDO in the tissues, resulting in an increase in Kyn content without any change in Trp content in those tissues. However, the exact reason why Kyn content increased despite the absence of any change in Trp content in the brain, kidney, lung, and spleen of +WIRS rat is unclear at the present time. Accordingly, further studies are needed to clarify the reason for the increase in Kyn content without any change in Trp content in the brain, kidney, lung, and spleen of rats exposed to WIRS.

IDO1 is known to be induced in various extrahepatic tissues and various cell types by pro-inflammatory cytokines such as INF-α, INF-γ, TNF-α, and IL-1, especially INF-γ [16–19]. IDO1 has been reported to be induced by IFN-α, INF-γ, and TNF-α in the brain of mice subjected to acute psychological stress [21]. It has also been shown that the levels of pro-inflammatory cytokines, such as TNF-α and IL-1β, are increased in the plasma and gastric mucosal tissue of rats exposed to WIRS [22–25]. Therefore, we examined changes in INF-γ, TNF-α, and IL-1β levels in the serum and liver of rats subjected to 6 h of WIRS (+WIRS) to verify whether INF-γ, TNF-α, and/or IL-1β contribute to IDO1 induction in the liver of the stressed rats. We found that +WIRS rats had increased levels of INF-γ in the serum and liver but that there were no increases in the levels of TNF-α and IL-1β in the liver even though serum TNF-α and IL-1β levels were increased. In an unpublished experiment we also observed that liver INF-γ levels in rats exposed to WIRS for 1.5 and 3 h are 1.6- and 1.9-fold, respectively, higher than those in the corresponding unstressed rats. Taken together, these findings suggest the possibility that increased INF-γ in the liver of +WIRS rats contributes to IDO1 induction in the tissue.

It is well known that liver TDO is induced via corticosteroid hormones secreted from adrenal glands through the hypothalamus–pituitary–adrenal axis in rats exposed to various types of stress [8–12]. Liver TDO induction in rats treated with glucocorticoid hormones occurs via glucocorticoid receptors in the tissue [43, 44]. Hirota et al. [44] demonstrated that a new glucocorticoid receptor species that differs from the typical glucocorticoid receptor is related to induction of TOD, but not to tyrosine aminotransferase, in the liver of rats treated with high-dose dexamethasone. Based on this finding, it may be possible to assume that the absence of any increase in TDO activity in the kidney and brain of the +WIRS rats in our study is due to the lack of just such a specific glucocorticoid receptor species involved in glucocorticoid hormone-mediated TOD induction in both tissues; however, it remains necessary to clarify the reason why TDO was not induced in the kidney and brain of the +WIRS rats. Based on these findings, we examined whether IDO1 induction did occur in the liver tissue of our +WIRS rats when the glucocorticoid signal via glucocorticoid receptors in the liver tissue was reduced by pretreatment with a glucocorticoid receptor antagonist, RU486. Pretreatment of the +WIRS rats with RU486

caused a further increase in increased serum corticosterone concentration and a reduction in increased serum glucose concentration. A similar further increase in increased plasma corticosterone concentration has been shown in RU486-pretreated rats subjected to restraint stress [45]. A similar reduction in increased blood glucose concentration has been reported in RU486-preteated rats subjected to physical stress or emotional stress [46]. It is known that interference with glucocorticoid binding to tissue in the hypothalamus by RU486 blocks normal negative feedback mechanisms, resulting in compensatory increases in serum glucocorticoid and corticotrophin (adrenocorticotrophic hormone) levels [47]. In our study RU486 pretreatment partially recovered the decrease in serum Trp concentration and the increase in serum Kyn concentrations found in +WIRS rats. This result indicates that glucocorticoid hormones secreted from adrenal glands through the hypothalamus–pituitary–adrenal axis are apparently involved in an enhancement of Trp catabolism in the body of +WIRS rats. RU486 pretreatment caused an apparent reduction in increased TDO activity with partial recoveries of decreased Trp and increased Kyn contents in the liver of the +WIRS rats, but it did not affect increases in IDO activity and INF-γ level in the liver of the these rats. These results suggest that the induction of IDO1 in the liver of +WIRS rats is independent of the induction of TDO in this tissue, leading to an enhancement of Trp catabolism in the liver. They also support the above-described possibility that IDO1 induction in the liver of +WIRS rats is caused via increased INF-γ in this tissue. Exposure to stress activates not only the hypothalamus–pituitary–adrenal axis, leading to the release of glucocorticoid hormones from the adrenal cortex, but also the sympathetic nerve–adrenal medullary axis, leading to the release of noradrenaline from sympathetic nerve endings and adrenaline from the adrenal medulla [48]. It has been reported that a single exposure of fasted rats to 6 h of WIRS increases plasma adrenalin and noradrenaline levels [49] and that the major source of hyperglycemia in fasted rats subjected to immobilization stress is gluconeogenesis due to adrenaline released from the adrenal medulla [50]. Catecholamines activate β-adrenergic receptors expressed on natural killer cells, T cells. B cells, and monocytes, resulting in the expression and release of pro-inflammatory cytokines, especially IFN-γ [51]. These findings may allow us to assume a possibility that increases in serum and liver INF-γ levels found in rats exposed to WIRS are caused through activation of the sympathetic nerve–adrenal medullary axis. However, it is unclear at the present time whether such an increase in INF-γ level through activation of the sympathetic nerve–adrenal medullary is critical for IDO1 induction in the liver of rats exposed to WIRS. Therefore, additional studies should be conducted to elucidate this matter.

In conclusion, our analysis of the liver, brain, lung, and spleen indicates that a single 6-h exposure of rats to WIRS (+WIRS) enhances Trp catabolism by inducing both IDO1 and TDO in the liver. They also suggest that IDO1 induction in the liver of +WIRS rats could be caused via increased INF-γ in this tissue. However, the exact mechanism for IDO1 induction in the liver of +WIRS rats is still unclear and, therefore, further studies are needed to clarify the exact mechanism for IDO1 induction in the liver of these rats.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest concerning this article.

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