ATRA Regulates Innate Immunity in Liver Ischemia/Reperfusion Injury via RARα/Akt/Foxo1 Signaling

Chen Zhong, a,b,8 Liyong Pu, a,b,8 Mingming Fang, c Jianhua Rao, a,b and Xuehao Wang*, a,b

a Key Laboratory on Living Donor Liver Transplantation, National Health and Family Planning Commission; Nanjing 210029, Jiangsu Province, P. R. China; b Department of Liver Surgery, First Affiliated Hospital of Nanjing Medical University; Nanjing 210029, Jiangsu Province, P. R. China; and c Department of Neurology, Jiangsu Province Hospital on Integration of Chinese and Western Medicine, Nanjing University of Chinese Medicine; Nanjing 210028, Jiangsu Province, China.

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All-trans retinoic acid (ATRA) has been proved to protect liver from ischemia/reperfusion (IR) injury, however, its mechanism is still unclear. This study is to investigate the mechanism of effect of ATRA on innate immunity in mice liver IR injury. Before operation, mice were gavaged by ATRA at 15 mg/kg/d for two weeks, and then the liver was underwent 70% ischemia (90 min) and reperfusion (6 h). Liver function was assessed by serum alanine aminotransferase (sALT), serum aspartate aminotransferase (sAST). Real-time PCR and Western blot were to detect the level of mRNA and protein. In vitro, RAW264.7 macrophages were treatment with ATRA (1 µM) or LE540 (5 µM, a retinoic acid receptor α (RARα) receptor antagonist) before lipopolysaccharide (100 ng/mL) stimulation. In vivo, ATRA protected the liver from IR injury by improving hepatocellular function (sALT and sAST), decreasing cell apoptosis and inhibiting inflammatory response (i.e., the level of toll-like receptor 4, transcription factor nuclear factor-xBp65, interleukin (IL)-1β, IL-6, and tumor necrosis factor-α). When RARα was blocked by LE540 in RAW264.7 macrophages, the inflammatory cytokines were enhancing, along with a decline of Akt phosphorylation but Forkhead box o (Foxo) 1, compared with the ATRA group. In summary, ATRA regulates in part the innate immunity to protect liver from IR injury by RARα/Akt/Foxo1 pathway.

Key words innate immunity; all-trans retinoic acid; retinoic acid receptor α; Forkhead box o 1

Hepatic ischemia/reperfusion (IR) injury is the common clinical problem during liver resection and transplantation. IR injury is also a significant factor in transplantation conditions, which can lead to early organ failure and increase the incidence of both acute and chronic rejection.1 Liver IR injury is the sterile immune response that has a biphasic pattern, including the acute phase is caused by activating Kupffer cells at 3–6 h, and subacute phase is resulting from neutrophil infiltration at 18–24 h.2–4 Hence, the liver inflammation response is of paramount importance factor, there has been proved that prevention of local immune activation consistently ameliorates the IR injury cascade.5 Multiple innate immune activation pathways have been identified, in particular, the toll-like receptor (TLR) 4/transcription factor nuclear factor-kappaB (NF-κB) pathway.5 Damage-associated molecular pattern molecules from damaged/stressed cells, including high mobility group box-1 protein, heat shock proteins, heparin sulfate and so on, activate TLRs, then propagate the inflammation response and lastly damage liver parenchymal cells.5

Forkhead box o (Foxo) proteins of the mammalian DAF-16-like transcription factors take part in many physiological and pathological processes, such as promote cell survival by resisting oxidative stress6–9 and suppress inflammatory cytokines under the control of Akt.10,11 Additionally, the disruption of the Akt/Foxo1 pathway would lead to Foxo accumulation, promote downstream gene expression and cause cell apoptosis in turn.12,13 Moreover, Akt/Foxo1 signaling was recently found to regulate innate immunity in liver IR injury.14,15

All-trans retinoic acid (ATRA), the main biologically active metabolite of vitamin A, has been widely applied in the treatment of acute promyelocytic leukemia. Recently, ATRA has been proved to ameliorate reactive oxygen species generation, activate the anti-oxidant defence system, and subsequently repress apoptosis.6,16 Meanwhile, ATRA also has been shown to mediate inflammatory responses by promoting inducible regulatory T cell and inhibiting NF-κB, TLRs and CD14 expression.17–19 Foxo proteins have been demonstrated that involve in regulating the spectrum of retinoid-mediated transcription and downstream-related cell activates,20 but the relationships among ATRA, Foxo proteins and liver IR injury are not well understood.

MATERIALS AND METHODS

Animals Male wild-type C57BL/6 mice (8–12 weeks old, Vital River Experimental Animal Co., P. R. China) were cared for according to the Chinese Association of Laboratory Animal Care and the standards for animal use and care set by the Institutional Animal Care Committee.

Liver IR Injury Model Mice were intra gastrically administered ATRA (15 mg/kg/d for 14 d, Sigma-Aldrich, Shanghai, China) before the operation. ATRA was dissolved in dimethyl sulfoxide (DMSO) and diluted in corn oil. The mouse model was partial (70%) hepatic warm ischemia followed by reperfusion.21 The operation mice received heparin (100 U/kg), and an atraumatic clip was used to occlude the arterial and portal venous blood supply to the cephalad lobes of the liver.
for 90 min, and then the clamp was removed. Mice were sacrificed at 6 h after reperfusion.

**Hepatocellular Function Assay** To analyze the degree of hepatocellular injury, serum alanine aminotransferase (sALT) and serum aspartate aminotransferase (sAST) levels were measured using an automated chemical analyzer (Olympus Automated Chemistry Analyzer AU5400, Japan).

**Histopathologic Study of Liver Tissue** Partial liver specimens were fixed with 10% neutral formaldehyde, and then embedded in paraffin. Liver sections (5 µm) were stained with hematoxylin and eosin and then scored as previously described.21)

**Cell Culture** RAW264.7 (ATCC, Manassas, VA, U.S.A.) mouse macrophage cells were maintained in Dulbecco’s minimum essential medium supplemented with 10% heat-inactivated fetal bovine serum. ATRA (1 µM) or LE540 (5 µM, Wako Pure Chemical Industries, Ltd., Osaka, Japan) was first added into the medium for 1 h, then lipopolysaccharide (LPS) (100 ng/mL; Sigma-Aldrich) was used to activate the cells.

**Real-Time RT-PCR Analysis** Total RNA was extracted using TRIzol reagent according to the manufacturer’s protocol (Invitrogen, Shanghai, China) and then reverse transcribed into cDNA by using a reverse transcriptase kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The primer sets were: interleukin (IL)-6 (sense: GAC TTC CAT CCA GTT GCC TTC T, anti-sense: TTT CTC ATT TCC ACG ATT TCC); tumor necrosis factor-α (TNF-α) (sense: CTC TGT GAA GGG AAT TTC CA); IL-1β (sense: GTG TTT TCC TTC TTG CCT CTG AT, anti-sense: GCT GCC TAA TGT CCC CTT GAA T); tumor necrosis factor-α (TNF-α) (sense: CTT TGT GAA GGA GGT GGT TGT, anti-sense: TCT TGT GTT TCT GAG TAG TTG TTG A); β-actin (sense: CTA CAA TGA GCT GGG TGT G, anti-sense: AAG GAA GCC TGG TGG AAG ATG GC).

**Western Blot Analysis** Proteins were extracted from liver samples and cell lysates, and the concentration was detected using a BCA Protein Assay Kit (Thermo Fisher Scientific, Shanghai, China). The proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotted onto polyvinylidene fluoride (PVDF) membranes (Millipore, U.S.A.). These membranes were blocked in non-fat dry milk (5% w/v) with Tris-buffered saline containing 0.1% Tween 20 (TBS-T) at 4°C overnight and then incubated with primary antibodies against retinoic acid receptor α (RARα) (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.), β-actin, Bcl-2, Cleaved caspase-3 (Cell Signaling Technology, Danvers, MA, U.S.A.), p-Akt, Foxol, TLR4, NF-κBp65 (Abcam, Shanghai, China). Following three washes with TBS-T, the membranes were incubated with peroxidase-conjugated secondary antibody (Cell Signaling Technology) for 1 h at room temperature. Bands were quantified by densitometry using the Quantity One software for image analysis.

**Statistical Analysis** Data are expressed as the mean±standard deviation (S.D.) and were statistically analyzed using one way ANOVA. Differences were considered statistically significant at probability level p<0.05.

**RESULTS**

**ATRA Ameliorates Liver IR Injury** ATRA was administered for two weeks, and then we examined the effect of the drug on liver IR injury. After 6 h of reperfusion, liver injury was evaluated by measuring the sALT and sAST levels.

### Table 1. Serum Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST) in Sham, Sham+ATRA, IR and IR+ATRA Groups

<table>
<thead>
<tr>
<th>Group</th>
<th>ALT (U/L)</th>
<th>AST (U/L)</th>
</tr>
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<tbody>
<tr>
<td>Sham</td>
<td>50±13</td>
<td>176±46</td>
</tr>
<tr>
<td>Sham+ATRA</td>
<td>60±17</td>
<td>171±39</td>
</tr>
<tr>
<td>IR</td>
<td>8080±363***</td>
<td>11642±2166***</td>
</tr>
<tr>
<td>IR+ATRA</td>
<td>6095±676***</td>
<td>8909±505***</td>
</tr>
</tbody>
</table>

Data were presented as mean±S.D., n=5/group; ***p<0.001 vs. the Sham group; ###p<0.001 vs. the IR group. Sham group and Sham+ATRA is equal to negative and positive control group in this study, respectively.

In the ATRA group, the protection was clear, with the sALT and sAST being lower than those in the IR group (Table 1). Additionally, we found no differences between the negative and positive control groups. Therefore, in the following experiments, we did not analyse results from the positive control group. Meanwhile, the area of hepatocyte necrosis and Suzuki scores in the ATRA group was less than that in the IR group, which was consistent with the change of liver function (Figs. 1C–D).

**ATRA Prevents Immune Responses and Apoptosis** RT-PCR was used to assess tissue expression of cytokine-chemokine genes including TNF-α, IL-1β and IL-6. After IR insult, the inflammatory cytokines were higher in the IR group in comparison with the negative control group (Fig. 1A). ATRA pretreatment, however, downregulated the mRNA levels of these cytokines (Fig. 1B). The results furtherly confirmed that ATRA protected liver from IRI, and the mechanism would be uncovered in the following study.

**RARα Is Involved in Mediating Akt/Foxol Signaling** Western blot analysis revealed that the levels of TLR4 and NF-κBp65 (Fig. 2) were inhibited by ATRA. The result indicated that ATRA regulated RARα activation to induce an anti-inflammatory effect, and this role maybe related to p-Akt and Foxol. This conclusion can be made because, compared with the IR group, the expression of p-Akt was upregulated whereas Foxol was downregulated in the ATRA group (Fig. 2), and Akt/Foxol is involved in the innate immune response.14,15)

**RARα Activation Attenuates the Inflammatory Response in Vitro** In Fig. 3, it showed that ATRA can inhibit the mRNA expression of TNF-α, IL-1β and IL-6 during LPS stimulation of RAW264.7 cells for 1 h. However, LE540, an inhibitor of RARα, increased the expression of these cytokines and aggravated the inflammatory response. The significant differences in expression levels of RARα between the ATRA and LE540 groups implied that RARα is involved in regulating innate immunity.

**RARα Activation Suppresses TLR4 Responses through the Akt/Foxol Pathway** To explain the mechanism of RARα in innate immunity, Akt/Foxol signaling may be considered to participate because Akt/Foxol has been shown to regulate inflammation and RARα mediates the transcription of Foxol.14,20) Furthermore, we found that LE540 reduced the level of p-Akt, upregulated the expression of Foxol, and then activated the TLR4/NF-κBp65 pathway by decreasing RARα expression (Fig. 4). In the ATRA group, RARα activation enhanced p-Akt, decreased the level of Foxol, and suppressed the expression of inflammation-related factors throughout the process of LPS treatment in comparison to the LPS group.
DISCUSSION

In this study, we investigated the influence of ATRA on mouse hepatic ischemia (90 min) and reperfusion (6h) injury. These results showed that ATRA regulated the innate immunity by TLR4/NF-κBp65 pathway, which was dependent on activating RARα/Akt/Foxo1 signaling.

Liver transplantation is the only effective therapy for patients with end-stage liver disease. However, the donor liver inevitably undergoes IR injury, and its severity will influence the function of the liver graft and even enhance the rate of acute and chronic rejection. Additionally, inflammation, which can be activated by TLR4, has been shown to play a key role in liver IR injury. Thus, the means of minimizing the TLR4-mediated inflammatory response in liver IR injury is very important for transplant patients.

ATRA, a biologically active metabolite of vitamin A, binds with its receptors to mediate the transcription of many target genes, including cell growth and differentiation, development, and homeostasis genes. ATRA can increase manganese superoxide dismutase with activation of p38 mitogen-activated protein kinase and Akt to protect the liver from IRI. Moreover, ATRA pretreatment suppresses the expression of NF-κBp65, which is associated with downregulation of TNF-α and IL-6 in liver IR injury. In this study, ATRA pretreatment alleviated the inflammation, decreased apoptosis and protected liver from IR injury lastly. These results were consistent with previous researches.

The Foxo family was first described as daf16 in Cae-norhabditis elegans, and Foxo factors inhibit cellular proliferation, transcribe antioxidant and stress response genes, and regulate insulin sensitivity. Further, Foxo1 has been...
shown to enhance TLR4 activation to mediate the inflammatory response in liver IR injury, and the Akt/Foxo1 signaling network can be regulated to activate the TLR4 pathway by nuclear factor erythroid 2-related factor 2.14,15) Our current results show that ATRA can inhibit Foxo1 expression, which is accompanied by enhanced phosphorylation of Akt. The decrease of Foxo1 facilitates cell survival by relieving inflammation and apoptosis.25) Class I phosphatidylinositol 3-kinase (PI3K) is the main enzyme responsible for the promotion of cell survival by Foxo1 28,29) because Akt1, a downstream effector of PI3K, diminishes the level of Foxo1.30–32)

Isotretinoin, a treatment for acne, triggers many cellular events that are related to Foxo1 regulation.33) ATRA increases PI3K activity and PI3K catalytic subunit p110 β protein expression,34) which is why ATRA regulates the activation of Akt. We also found that ATRA promotes autophagy to alleviate liver injury caused by IR through the Foxo3/Akt/Foxo1 pathway.21) In our study, the activation of RARα by ATRA led to a decrease of Foxo1 and changed apoptosis-associated proteins, i.e., upregulation of Bcl-2 and downregulation of cleaved caspase-3. At the same time, the expression levels of TLR4 and NF-κBp65 were diminished. Therefore, we assumed that RARα plays an important role in regulating innate immunity. To further verify this hypothesis, LE540 was used to inhibit RARα. In turn, the expression of Foxo1 was found

**Fig. 2. RARα is Involved in Mediating Akt/Foxo1 Signaling**

The expression levels of RARα, p-Akt, Foxo1, TLR4, NF-κBp65 and β-actin were detected by Western blotting, and these proteins were normalized to the negative control group. Mean±S.D., n=5/group, **p<0.01 and ***p<0.001 vs. the sham group; **p<0.01 and ***p<0.001 vs. the IR group; sham group is equal to negative control group in this study.

**Fig. 3. RARα Activation Attenuated the Inflammatory Response in Vitro**

After ATRA or LE540 pretreatment for 24h, cells were preincubated with LPS and harvested at 1h. TNF-α, IL-6 and IL-1β mRNA levels were analyzed, and the ratios of these indicators were calculated. Mean±S.D., **p<0.01 vs. the DMSO group; "p<0.05 and ""p<0.01 vs. the LPS group; """"p<0.001 vs. the LPS+ATRA group.
to increase, and the inflammatory response was more serious. Hence, we concluded that ATRA was involved in regulating innate immunity. This pathway was controlled by RARα/Akt/Foxo1 signaling. However, whether Foxo3a participates in this pathway should be examined in the further studies.

In conclusion, we first demonstrated a novel mechanism of ATRA in downregulating the TLR4/NF-κBp65 pathway in liver IR injury. This protective effect relies on activation of the RARα/Akt/Foxo1 pathway. In addition, this article provides a promising future for ATRA in the clinic.

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Conflict of Interest The authors declare no conflict of interest.

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