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In vitro Treatment of Mouse and Human Cells with Endogenous Ligands for Activation of the Aryl Hydrocarbon Receptor

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[Abstract] Activation of the aryl hydrocarbon receptor (AHR) by endogenous ligands has been implicated in a variety of physiological processes such as cell cycle regulation, cell differentiation and immune responses. It is reported that tryptophan metabolites, such as kynurenine (Kyn) and 6-formylindolo(3,2-b)carbazole (FICZ), are endogenous ligands for AHR (Stockinger *et al*., 2014). This protocol is designed for treatment with Kyn or FICZ in mouse embryonic fibroblasts (MEFs) or primary peripheral monocytes.
Keywords: Aryl hydrocarbon receptor, Kynurenine, 6-formylindolo(3,2-b)carbazole, Tryptophan, TCDD-inducible poly(ADP-ribose)polymerase

[Background] Tryptophan metabolites such as Kyn and FICZ are endogenous ligands for AHR under physiological conditions. Kyn is generated by tryptophan-2,3-dioxygenase (TDO) and/or indoleamine-2,3-dioxygenases 1 and 2 (IDO1/2) and contributes to the suppression of antitumor response and malignant progression (Stockinger *et al*., 2014). FICZ is produced by exposure of L-tryptophan to ultraviolet B irradiation and is involved in many biological processes (Smirnova *et al*., 2016). In the adaptive immune system, FICZ is shown to promote Th17 cell response (Stockinger *et al*., 2014). It has also been shown that innate interferon response during viral infection is suppressed by treatment with these endogenous AHR ligands (Yamada *et al*., 2016). In order to evaluate the effect of AHR activation by treatment with these ligands, tryptophan-free culture medium and dialyzed FBS are used to cultivate cells under tryptophan-free conditions (Opitz *et al*., 2011). The detection of TCDD-inducible poly(ADP-ribose)polymerase (*TIPARP*) (Ma, 2002), one of the AHR-inducible genes, is analyzed to verify ligand-induced AHR activation.

Materials and Reagents

1. 0.1-10 µl pipet tips (Thermo Fisher Scientific, Thermo Scientific™, catalog number: QSP#TF104)
2. 1-200 µl pipet tips (Corning, catalog number: 4845)
3. 100-1,000 µl pipet tips (Corning, catalog number: 4846)
4. 15 ml centrifuge tubes (Corning, Falcon®, catalog number: 352096)
5. 50 ml centrifuge tubes (Corning, Falcon®, catalog number: 352070)

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6. 50 ml syringe (NIPRO, catalog number: 4987458089534)
7. 0.20 μm sterilizing filter (Advantec MFS, catalog number: 25CS020AS)
8. Falcon 12-well tissue culture plate (Corning, Falcon®, catalog number: 353043)
9. Seamless cellulose tubing (EIDIA, catalog number: 410490033)
10. 96-well fast plate (NIPPON Genetics, catalog number: 38801)
11. qPCR adhesive seal (NIPPON Genetics, catalog number: 4Ti-0560)
12. Mouse embryonic fibroblasts (MEFs) of C57/B6 origin (E13.5) (Bryja *et al*., 2006)
13. CD14 microbeads, human (Miltenyi Biotec, catalog number: 130-050-201)
14. Primary human peripheral monocytes, which are Isolated from peripheral blood of healthy volunteers by magnetic-activated cell sorting with CD14 microbeads according to the manufacturer's instructions
15. ISOGEN (Nippon Gene, catalog number: 311-02501)
16. Nuclease free-H_2O (as an accessory reagent of ReverTra Ace qRT-PCR Kit) (TOYOBO, catalog number: FSQ-101)
17. DNase I (Thermo Fisher Scientific, Invitrogen™, catalog number: 18068015)
18. EDTA
19. ReverTra Ace qRT-PCR Kit (TOYOBO, catalog number: FSQ-101)
20. SYBR Premix Ex Taq (2x) (Tli RNase H Plus) (Takara Bio, catalog number: RR420)
21. ROX reference dye (50x) (Thermo Fisher Scientific, Invitrogen™, catalog number: 12223-012)
22. Primers for amplification of mouse TIPARP cDNA by quantitative PCR (Sigma-Aldrich):
    Forward: 5'-GCCAGACTGTGTAGTACAGCC-3'
    Reverse: 5'-GGGTTCCAGTTCCCAATCTTTT-3'
23. Primers for amplification of mouse ACTB cDNA by quantitative PCR (Sigma-Aldrich):
    Forward: 5'-AGTGTGACGTTGACATCCGTA-3'
    Reverse: 5'-GCCAGAGCAGTAATCTCCTTCT-3'
24. Primers for amplification of human TIPARP cDNA by quantitative PCR (Sigma-Aldrich):
    Forward: 5'-GTTGGGGACCAGATACCGGA-3'
    Reverse: 5'-CTGGGTGCAAAAGATCAGTCT-3'
25. Primers for amplification of human GAPDH cDNA by quantitative PCR (Sigma-Aldrich):
    Forward: 5'-CATGAGAAGTATGACAACAGCCT-3'
    Reverse: 5'-AGTCCTTCCACGATACCAAAGT-3'
26. NaCl
27. $NaH_2PO_4 \cdot H_2O$
28. KCl
29. $CaCl_2$
30. $MgSO_4 \cdot 7H_2O$
31. $Fe(NO_3)_3 \cdot 9H_2O$
32. L-arginine·HCl
33. L-histidine·HCl·H_2O

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34. L-isoleucine
35. L-leucine
36. L-lysine·HCl
37. L-methionine
38. L-phenylalanine
39. L-threonine
40. Glycine
41. L-valine
42. L-cysteine·HCl·H_2O
43. L-serine
44. L-tyrosine
45. Choline bitartrate
46. Folic acid
47. D-Ca pantothenate
48. Myo-Inositol
49. Niacinamide (Nicotinamide)
50. Pyridoxal·HCl
51. Thiamine·HCl
52. Riboflavin
53. D-glucose
54. Sodium pyruvate
55. Phenol Red Na
56. Succinic acid
57. Disodium succinate
58. Dulbecco's modified Eagle's medium (DMEM) (NISSUI PHARMACEUTICAL, catalog number: 05919)
59. L-glutamine (Thermo Fisher Scientific, Gibco™, catalog number: 21051-024)
60. $NaHCO_3$ (KANTO KAGAKU, catalog number: 37116-00)
61. Fetal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10437-028)
62. Stock solutions of L-kynurenine (Kyn) (Sigma-Aldrich, catalog number: K8625) and 6-formylindolo(3,2-b)carbazole (FICZ) (Enzo Life Sciences, catalog number: BML-GR206-0100)
63. Phosphate-buffered saline (PBS) (pH 7.4) (NISSUI PHARMACEUTICAL, catalog number: 05913)
64. Dimethylsulfoxide (DMSO) (Dojindo Molecular Technologies, catalog number: SP10)
65. Isopropanol (NACALAI TESQUE, catalog number: 29113-53)
66. Ethanol (99.5%) (NACALAI TESQUE, catalog number: 14713-95)
67. Chloroform (KANTO CHEMICAL, catalog number: 07278-00)
68. Tryptophan-free (Trp-free) DMEM (Cell Science & Technology Institute, special order) (see Recipes)

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69. DMEM (FBS+) (see Recipes)
70. Trp-free DMEM (FBS+) (see Recipes)
71. Kyn stock solution
72. FICZ stock solution

Equipment

1. Pipettes (PIPETMAN P2) (Gilson, catalog number: F144801)
2. Pipettes (PIPETMAN P20) (Gilson, catalog number: F123600)
3. Pipettes (PIPETMAN P1000) (Gilson, catalog number: F123602)
4. Bio clean bench (Hitachi, model: 1305BNG3-AG)
5. Labnet VX100 vortex (Labnet International, catalog number: 13111-LV2)
6. 37 °C and 5% CO_2 cell culture incubator (WAKENBTECH, catalog number: 9000EX)
7. ABI StepOnePlus™ Real-Time PCR systems (Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4379216)

Procedure

1. Treatment with Kyn or FICZ (each sample is usually prepared in triplicate to assess reproducibility)
   a. Seed MEFs or primary human monocytes to 12-well plate as 1 x 10^6 cells/well with 500 µl of DMEM (FBS+) and culture them at 37 °C in a 5% CO_2-incubator.
   b. After 24 h, MEFs are washed once with 1 ml of pre-warmed PBS, and incubated with 500 µl of Trp-free DMEM (FBS+) for 24 h at 37 °C. This step is performed to cultivate cells under Trp-free conditions. This Trp-free culturing does not affect the growth of MEFs at least under these conditions.
   c. Remove medium, and incubate cells with 500 µl of Trp-free DMEM (FBS+) containing Kyn (0, 50, 100 or 200 µM) or FICZ (0, 0.1, 1 or 25 nM) for 2 h at 37 °C.
2. To evaluate the effect of AHR ligands, the mRNA induction of TIPARP, one of the AHR-inducible genes, is analyzed by qRT-PCR as below:
   a. At 2 h after treatment of AHR ligands, cells are washed with PBS and dissolved by directly adding 500 µl of ISOGEN. Total RNA is then extracted according to the manufacturer's protocol and adjusted to 1 µg/µl with nuclease free-H_2O.
   b. In this step, total RNA is purified by degrading contaminated DNA with DNase I treatment. Make a mixture in the following order:

Isolated total RNA (1 µg/µl)	1.0 µl
DNase I (1 U/µl)	1.0 µl
10x DNase I reaction buffer	1.0 µl
Nuclease free-H_2O	7.0 µl
Total	10.0 µl

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c. Incubate the mixture samples at 37 °C for 15 min.
d. Add 1 µl EDTA to each sample and incubate the samples at 65 °C for 10 min. This step is needed to stop the activity of DNase I.
e. To next synthesize cDNA with a ReverTra Ace qRT-PCR Kit, make a mixture with each premixed reagent that is included in the kit, in the following order:

DNase I/EDTA-treated samples (from step 2d as above)	7.0 µl
Enzyme mix	0.5 µl
Primer mix	0.5 µl
5x RT buffer	2.0 µl
Total	10.0 µl

f. Incubate the mixture samples at 37 °C for 15 min, and then incubate them at 98 °C for 5 min.
g. Make a 4-fold dilution of the cDNA samples with nuclease free-H_2O for each qPCR template.
h. Expression levels of reverse-transcribed mRNA, that is, cDNA, in each sample are next evaluated by qPCR analysis, wherein SYBR, a DNA-binding fluorescent dye, is used for the measurement of the levels of amplified DNAs, while ROX is used as a reference dye. Set up the following reaction mixture to amplify cDNA:

Nuclease free-H_2O	7.8 µl
2x SYBR Premix Ex Taq	10.0 µl
50x ROX reference dye	0.4 µl
Forward primer (10 µM)	0.4 µl
Reverse primer (10 µM)	0.4 µl
Diluted cDNA sample	1.0 µl
Total	20.0 µl

Note: Use 1 µl of nuclease free-H_2O or no-RT samples instead of a DNA sample as a negative control to confirm the procedure works as expected.

i. The PCR plate is covered with an adhesive transparent cover, and then centrifuged shortly.
j. Set the plate in the real-time instrument and start the real-time PCR following the program as below:

Holding stage	95 °C	10 sec		1 cycle
Cycling stage	95 °C	5 sec	Data collection	45 cycles
	60 °C	30 sec		
	95 °C	15 sec		
Melt curve stage	60 °C	60 sec	Data collection during 60 °C → 95 °C	1 cycle
	95 °C	15 sec		

Data analysis

1. TIPARP mRNA levels are normalized to the mRNA expression levels of ACTB or GAPDH for each sample by ΔΔCt methods (Schmittgen and Livak, 2008).
2. Statistical significance between two samples is determined by Student's *t*-test.

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Recipes

1. Tryptophan-free DMEM

   Table 1. Composition of Trp-free DMEM. This is prepared by special order (Cell Science & Technology Institute).

Composition	Concentration (mg/L)	Composition	Concentration (mg/L)
NaCl	6,400.00	L-cysteine·HCl·H_2O	70.30
$NaH_2PO_4·H_2O$	124.20	L-serine	42.00
KCl	400.00	L-tyrosine	72.00
$CaCl_2$	200.00	Choline bitartrate	7.20
$MgSO_4·7H_2O$	200.00	Folic acid	4.00
$Fe(NO_3)_3·9H_2O$	0.10	D-Ca pantothenate	4.00
L-arginine·HCl	84.00	myo-Inositol	7.20
L-histidine·HCl·H_2O	42.00	Niacinamide(Nicotinamide)	4.00
L-isoleucine	104.80	Pyridoxal·HCl	4.00
L-leucine	104.80	Thiamine·HCl	4.00
L-lysine·HCl	146.00	Riboflavin	0.40
L-methionine	30.00	D-glucose	1,000.00
L-phenylalanine	66.00	Sodium pyruvate	110.00
L-threonine	95.00	Phenol Red Na	5.00
Glycine	30.00	Succinic acid	106.00
L-valine	93.60	Disodium succinate	16.21

2. DMEM (FBS+)
   DMEM is supplemented with 4 mM L-glutamine, 0.1% $NaHCO_3$ and 10% heat inactivated FBS
3. Trp-free DMEM (FBS+)
   a. To deplete tryptophan from FBS, FBS is dialyzed against 100-fold volumes of PBS at 4 °C for 24 h using seamless cellulose tubing with exchange of the dialysis solution
   b. Trp-free DMEM is supplemented with 4 mM L-glutamine, 0.1% $NaHCO_3$ and 10% heat inactivated and dialyzed FBS
4. Kyn stock solution
   Kyn is dissolved at 25 mM in PBS
5. FICZ stock solution
   FICZ is dissolved at 10 mg/ml (35.2 mM) in DMSO

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