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Abstract

In this work we demonstrate that Caco-2 cell treatment with WY-14643 (a potent PPARα agonist) causes an increase in AhR expression. Luciferase assays and directed mutagenesis experiments showed that induction mainly occurred at transcriptional level and involved a PPRE site located within the AhR promoter. These results were further confirmed by the use of PPARα knockout mice in which AhR induction by WY14643 was abrogated. In addition to CYP1 regulation, AhR has been described as being involved in inflammation, so we also studied the effect of AhR regulation by PPARα on the expression of some inflammation target genes. 3-Methylcholanthrene (a potent AhR agonist) increased the expression (mRNA) of the major inflammatory targets IL-1β and MMP9. WY-14643 co-treatment abrogated the 3-methylcholanthrene proinflammatory effect. Hence the anti-inflammatory effect of PPARα overrides the pro-inflammatory effect of AhR.

Introduction

Our daily diet exposes us to an array of compounds that include nutrients such as fatty acids and cooking by-products such as polycyclic aromatic hydrocarbons (PAHs), which can modulate the expression of various target proteins, including transcription factors. The gut can thus be considered as a primary target organ, yet the effects of such coexposure on its biological functions are little known.

Ah receptor (AhR) can be activated by food products such as PAHs formed during fat pyrolysis, and indoles found in cruciferous vegetables. The role of AhR in the regulation of CYP1 expression and some phase 2 enzymes of xenobiotic metabolism has been extensively studied, owing to the role of CYP1 in carcinogenesis [1]. CYP1 catalyses the bioactivation of numerous procancerogens including PAHs and arylamines, which are thought to be involved in the aetiology of colon cancers. Recent data have suggested that AhR is also involved in the inflammation process. The transcription factor NFκB is a central mediator of the immune and inflammatory response [2]. It is regulated through the binding of inhibitory molecules referred to as the IκB proteins. On activation, NFκB rapidly enhances the expression of proinflammatory genes such as cytokines and cell adhesion molecules. A molecular interaction has been described between AhR and the NFκB RelA sub-unit, forming a heterodimer able to activate the NFκB responsive elements [3]. Using mice with triple knockout of receptors for TNFα, TNFβ and IL-1α and IL-1β, it was found that the hepatic inflammation induced by dioxins was mediated by "IL-1-like" cytokines [4].

Also, PPARα, in addition to its role as an important lipid sensor and regulator of cellular energy-harvesting metabolism, has been reported to be involved in the physiopathology of the inflammatory process, notably by repressing NFκB and activator protein-1 (AP-1) signaling [5].

We previously reported that activation of PPARα by its potent ligand WY14643 (WY) led to an induction of CYP1A1 expression in Caco-2 cells [6]. Also, when cells were coexposed to WY and 3-methylcholanthrene (3MC) (a potent PAH agonist of AhR), we observed a potentiation of CYP1A1 inducibility through AhR [7]. This effect was associated with an induction of AhR expression by PPARα, but the molecular mechanisms involved in this induction were not studied.

PPARα and AhR might therefore work together to induce CYP1A1, but exert opposite effects towards inflammation. The effects of coexposure to PPARα and AhR ligands on the inflammatory state remain to be clarified.

The aim of our work was to elucidate the molecular mechanism involved in AhR induction by PPARα in Caco-2 cells and to evaluate the effects of coexposure to ligands of these transcription factors on the expression of some inflammation genes.

Materials and methods

Chemicals. WY was from VWR International (Fontenay-sous-Bois, France); 3MC, dimethylsulphoxide (DMSO) and bovine serum albumin (BSA) were from Sigma (Saint-Quentin-Fallavier, France).

Cell culture. Caco-2 cells from human colorectal adenocarcinoma [8] were a generous gift from Dr. Le Bivic (IBDM, Marseille, France). Cells were maintained in Dulbecco's modified Eagle's medium (Eurobio, Les Ulis, France) supplemented with 10% foetal bovine serum (FBS) (Eurobio), 2 mM L-glutamine (Life Technologies, Cergy-Pontoise, France), 1% non-essential amino acids (Life Technologies), 100 UI/ml penicillin and 100 µg/ml streptomycin (Life Technologies), at 37 °C and 9% CO_2.

As serum contained PPARα agonists, 12 h before treatments the medium was removed and replaced with a serum-free medium containing 0.2% BSA.

For the evaluation of AhR expression, cells were treated for 6 h with increasing concentrations of WY (10-400 µM). To evaluate the effect of coexposure to WY and 3MC on mRNA level of CYP1A1 and some inflammation target proteins (IL1β, ICAM1, MMP9), cells were pre-treated for 6 h with WY (200 µM) and treated for 6 h with a mixture of WY (200 µM) and 3MC (1 µM).

For fluorescent imaging and luciferase assay, cells were treated as described above, for 24 h and 48 h respectively.

RNA extraction and real time PCR. Total cellular RNA was isolated from Caco-2 cell cultures with Nucleospin RNAII (Macherey Nagel, France). Total RNA (1 µg) was reverse-transcribed using Moloney murine leukemia virus reverse-transcriptase (Invitrogen, Cergy-Pontoise, France) and random primers at 42°C for 1 h.

Human AhR or CYP1A1 mRNA expression normalised to *β*2-microglobulin expression were determined using the LightCycler System (Roche Diagnostics, France) and the Faststart DNA master SYBRGreen I kit (Roche Diagnostics). The primers for AhR, CYP1A1 and *β*2-microglobulin were as follows:

HumAhR-F: 5'-CAGAAAACAGTAAAGCCAATCC-3'

HumAhR-R: 5'-AATACAAAGCCATTCAGAGCC-3'

Hum*β*2m-F: 5'-CCGACATTGAAGTTGACTTAC-3'

Hum*β*2m-R: 5'-ATCTTCAAACCTCCATGATG-3'

PCR was performed with 4mM $MgCl_2$, 0.25 µM of each primer and LightCycler FastStart DNA Master SYBR Green I mix in a total volume of 16 µl. Cycling conditions were as follows: 10 min denaturing at 95°C, followed by 45 cycles of 10 s

denaturing at 95°C, 8 s primer annealing at 55°C and 8 s of fragment elongation at 72°C. The melting curve was analysed with the LightCycler software and quantification was carried out with RelQuant software (Roche Diagnostics).

Murine AhR and human ICAM-1, IL1β, MMP9 mRNA expression normalised to β-actin expression were determined using the Mx3005P QPCR System (Stratagene, France) and the LightCycler 480 Probe Master (Roche Diagnostics). The reaction was performed as recommended by the supplier. The PCR efficiency was 90 ± 10%. The primers were designed using the assay design centre of the UPL website (www.universalprobelibrary.com). The primers sequences and the UPL probes used were as follows:

MusAhR-F: 5'-TGCACAAGGAGTGGACGA-3'

MusAhR-R: 5'-AGGAAGCTGGTCTGGGGTA-3'

UPL Probe used for MusAhR: #27

MusActin-F: 5'-AAGGCCAACCGTGAAAAGAT-3'

MusActin-R: 5'-GTGGTACGACCAGAGGCATAC-3'

UPL Probe used for MusActin: #56

HumICAM1-F: 5'-CCTTCCTCACCGTGTACTGG-3'

HumICAM1-R: 5'-AGCGTAGGGTAAGGTTCTTGC-3'

UPL Probe used for HumICAM1: #71

HumIL1β-F: 5'-AACAGGCTGCTCTGGGATT-3'

HumIL1β-R: 5'-TGGCTGCTTCAGACACTTGA-3'

UPL Probe used for HumIL1β: #41

HumMMP9-F: 5'-TGTACCGCTATGGTTACACTCG-3'

HumMMP9-R: 5'-GCCCCAGAGATTTCGACTC-3'

UPL Probe for HumMMP9: #53

HumActin-F: 5’-CCCAGCACAATGAAGATCAA-3’

HumActin-R: 5’-CGATCCACACGGAGTACTTG-3’

UPL Probe for HumActin: #63

AhR immunostaining. Caco-2 cells were plated on four-well/chamber Nunc® permanox slides and exposed for 24 h to 200 µM of WY. Immunostaining was performed as described elsewhere [9], using as primary antibody a rabbit polyclonal antibody raised against human AhR (sc-5579, Santa Cruz Biotechnology, Germany) (1/300), and as secondary antibody a goat anti-rabbit IgG-FITC (Santa Cruz Biotechnology) (1/200). Each image was obtained with a Leica DMIRBE microscope.

AhR promoter luciferase assays. Fragments of 2.4 and 2.7 kb of the human AhR gene 5′-flanking region were subcloned into the pGL3-enhancer luciferase vector (Promega, France) using *Kpn* I and *Xho* I sites, as previously described [10]. The following primers were used:

AhR-Prom(-2103/-2077): 5’-ACCATGTTCCACAGTGCCAATACAGGA-3’

AhR-Prom(-1764/-1738): 5’-TAGGATTTTTTTAAATCACTAAATTAA-3’

AhR-Prom(+637/+616): 5’-CAGCCGACGGCGGCGGCTACTC-3’

Restriction enzyme digestion and DNA sequencing were used to confirm the sequence of the promoter constructs.

Cells in six-well plates were grown to 50% confluence before transfection. Transient transfections were performed by lipofection (lipofectin, Life Technologies) in a serum- and antibiotic-free medium containing 2% L-glutamine, with 0.5 µg of p3.1 (containing the –1764/+637 region of AhR gene) or p3.48 (containing the –2103/+637 region of the AhR gene). After 48 h treatment with 200 µM of WY, the luciferase activity was evaluated using the Luciferase Assay System from Promega (Promega).

Site-directed mutagenesis of PPRE site of the AhR promoter. PPRE mutation of p3.1 and p3.48 was introduced using the QuickChange site-directed mutagenesis kit (Stratagene) to obtain the p3.1(ΔPPRE) and the p3.48(ΔPPRE). Sense and antisense primers were used for mutagenesis. The sense primers were as follows (the PPRE core is underlined and bold face indicates nucleotides mutated compared with the wild type sequence (Wt)):

Wt: 5'-GCTCTTCTAAAATTAAAACAAAGATCATCGACCACAAAACACAATTG-3'

Mut-F : 5'-GCTCTTCTAAAATTAAAACAA**CA**ATCATCGACCACAAAACACAATTG-3'

Presence of the mutations was verified by DNA sequencing. Cells were transfected by the p3.1, or p3.48, or p3.1(ΔPPRE), or p3.48(ΔPPRE) and after a 48 h treatment with WY (200 µM), luciferase activity was evaluated as described above**.**

Cloning of AhR PPRE sequence. Sense and antisense oligonucleotides containing a tandem of two AhR PPRE sites were synthesised with addition of *Kpn* I and *Xho* I opened restriction sites at the 5' and 3' ends respectively. After annealing, these two oligonucleotides were cloned in the corresponding sites of the pGL3 Promoter vector containing a SV40 promoter. This construction was named pPPRE-AhR-Luc. The forward oligonucleotide used was: 5'-**C**TTCTAAAATTAAAACAAAGATCATCGACCACAACTTCTAAAATTAAAACAAAG ATCATCGACCACAA**C**-3', and the reverse oligonucleotide was: 5'-**TCGAG**TTGTGGTCGATGATCTTTGTTTTAATTTTAGAAGTTGTGGTCGATGATC TTTGTTTTAATTTTAGAAG**GTAC-3'**. The PPRE sites are underlined and *Kpn* I and *Xho* I digested restriction sites are bold face.

Mice and treatments. Ten-week-old PPARα-deficient mice with a C57BL/6J genetic background [11] were bred at the INRA transgenic rodent facility (INRA, Toulouse, France). Age-matched male C57BL/6J mice were obtained from Charles

River (Les Oncins, France) and were acclimatised to local animal facility conditions for 2 weeks. Mice were treated *per os* for a week with 30 mg/kg WY or vehicle (three animals per group). *In vivo* studies were conducted under European Union guidelines for the use and care of laboratory animals and were approved by the institutional ethics committee.

Statistical analysis. All experiments were repeated at least three times. Means ± S.D. were calculated for each group. Statistical significance was determined using Tukey's multiple comparison test, except for AhR PPRE luciferase assays where the Mann-Whitney test was performed.

Results

Effects of WY exposure on AhR expression in Caco-2 cells. As shown in Figure 1A, treatment of Caco-2 cells with increasing amounts of WY led to an induction of AhR expression, with a maximum effect with 100 µM WY (3.5-fold, $p < 0.01$). The transcript induction was associated with an increase in protein expression (Fig. 2) in the cytoplasm and nucleus.

Effects of WY treatment on colic expression of AhR in wild type and PPARα knockout mice. To verify *in vivo* that WY exposure leads to an induction of AhR expression, wild type mice and PPARα knockout mice were treated with 30 mg/kg of WY. As shown in Figure 1B, WY enhanced AhR mRNA expression in the colon of wild type mice (about 2-fold, $p < 0.01$), but not in PPARα knockout mice.

Effects of WY exposure on AhR promoter activity in Caco-2 cells. To check the transcriptional origin of AhR induction by WY, Caco-2 cells were transiently transfected with p3.1 or p3.48. As shown in Figure 3A, WY efficiently induced the transactivation of the AhR wild type promoter (2.5-fold with p3.1, $p < 0.01$; 1.6-fold

with p3.48, $p < 0.01$). The greatest induction observed with p3.1, containing the -1764/+637 region of AhR gene, is consistent with the literature, the region between -2142 and -1764 of human AhR being reported to contain negative regulatory elements [10]. The mutation of the putative PPRE site located between -1282 and -1270 of human AhR promoter abrogated luciferase induction, demonstrating that this PPRE is functional and involved in AhR induction mediated by WY. Also, when luciferase expression was directly driven by human AhR PPRE, WY treatment caused an increase in luciferase [Fig. 3B].

Effects in Caco-2 cells of co-exposure to WY and 3MC on the expression of genes coding for some inflammation proteins. Figure 4 showed the effect of 3MC, WY, and 3MC+WY on some inflammation target proteins. ICAM1 mRNA level was not modified by 3MC and 3MC+WY, WY alone reduced its expression by about 50% ($p < 0.01$). IL1β mRNA was increased by 3MC (2.5-fold, $p < 0.01$), was slightly reduced by WY (-30%), and was not modified by 3MC+WY. MMP9 transcript was induced by 3MC (3.9-fold, $p < 0.01$) and slightly enhanced by WY and 3MC+WY (about 1.5-fold).

Discussion

Substances in the environment, including the diet, exert their biological effects by modulating the activity of transcription factors. We previously reported that PPARα activation potentiated CYP1A1 induction mediated by the AhR pathway [7], but the molecular events involved in this effect were not known.

Here we find that this potentiation is probably partly due to a transcriptional activation of AhR expression by PPARα ligand (WY). This agrees with the results we obtained

with PPARα KO mice, in which we did not observe any colic induction of AhR after WY treatment.

Analysis of human AhR promoter (Mathinspector [12]) revealed one putative PPRE site located at positions -1282/-1270. This PPRE seems to play an important role in AhR expression as its mutation led to the suppression of AhR promoter activation by WY, and as a construction containing only the PPRE-AhR sequences can be activated by WY.

AhR has been largely studied owing to its central role in the bioactivation of many environmental procarcinogens such as PAHs and arylamines. The AhR pathway is thus commonly viewed as an adaptive response toward xenobiotic agents. Recent data argues that AhR is also involved in normal physiology and development [13]. AhR activation leads to inflammation though the induction of IL-1β like pro-inflammatory cytokines [4, 14, 15, 16, 17], probably via NFκB [3] or AP-1 [18]. On the other hand, the anti-inflammatory activity of PPARα through the repression of NFκB and AP-1 is well known [5]. Since AhR and PPARα exert opposite effects towards inflammation, we examined the effects of coexposure to 3MC and WY on some inflammation target proteins.

We showed that 3MC treatment induces the expression (mRNA) of the potent pro-inflammatory cytokines IL-1β (Fig. 4). Similar results obtained after PAH or dioxin exposures are described in other cell types, such as macrophages [17], fibroblast-like synoviocytes [15], and keratinocytes [16]. This induction was described as being mediated by AhR [16]. Another well-known pro-inflammatory cytokine, TNFα, is also reported to be upregulated by PAH and dioxins, through the ERK pathway and not the AhR one [19]. In our conditions, we failed to detect any TNFα mRNA in Caco-2 cells, either before or after 3MC exposure (data not shown). WY did not significantly

modify IL-1β mRNA levels compared with control cells, but abrogated the inductive effect of 3MC (Fig. 4). Although the upregulation of AhR expression induced by WY may lead, as observed with CYP1A1, to a potentiation of IL-1β induction by 3MC, our data demonstrate that the anti-inflammatory effect of PPARα overrides the pro-inflammatory effect of AhR.

The upregulation of IL-1β is associated with the induction of various targets, including cytokines, cellular adhesion proteins and metalloproteinases (MMP). MMPs degrade extracellular matrix (ECM) components and activate growth factor, thereby contributing to physiological events (tissue remodelling in pregnancy, wound healing, angiogenesis) and pathological conditions (cancer, arthritis, periodontis) [20]. Among the different MMPs expressed in the intestine, we studied MMP9 because it is largely involved in intestinal inflammation [21] and upregulated by AhR activation [22]. Results obtained for MMP9 are comparable to those obtained for IL-1β (Fig. 4). 3MC dramatically induces MMP9 mRNA. WY alone does not significantly modify it, but suppresses the inductive effect of 3MC. MMPs were initially characterised as proteases that could degrade the ECM, but they are now known to increase cancer-cell growth, migration, invasion, metastasis and angiogenesis [23]. Chemical carcinogenesis induced by AhR ligands has been mainly associated with the induction of CYP1 family [1]. Taken together, these data suggest that AhR-mediated chemical carcinogenesis, apart from CYP1, may also involve MMP upregulation.

ICAM-1 is another major inflammatory protein that functions in cell-cell and cell-matrix adhesive interactions. These interactions are critical for the transendothelial migration of leucocytes and the activation of T cells, and ICAM-1 is associated with a variety of inflammatory diseases [24]. As shown in Figure 4, 3MC does not modify ICAM-1 mRNA level. An increase in ICAM-1 expression was

described in dendritic cells from TCDD-treated mice, requiring the presence of AhR [25]. The lack of ICAM-1 induction in intestine epithelial Caco-2 cells after 3MC exposure was probably due to specific cell/tissue regulation, and further data are required to confirm this hypothesis. As previously described *in vivo* in mice [26], WY was able to repress ICAM-1 expression.

Our diet contains nutrients and cooking by-products that can modulate intestinal physiology, notably through the activation and repression of transcription factors. It has been demonstrated that oxidised omega-3 fatty acids can inhibit NFκB activation, via a PPARα-dependent pathway [27]. Consumption of marine fish oil has been reported to improve the prognosis of inflammatory disorders, including inflammatory bowel disease (IBD) [28]. The effect of the activation of AhR, notably by PAH, on the inflammatory state of the intestine was not well known. Our data obtained *in vitro*, suggest that PAH can induce an inflammation *in vivo* through the increase in proinflammatory cytokines, such as IL-1β. This hypothesis is strengthened by epidemiological data suggesting that active cigarette smoking, which leads to PAH exposure, is associated with the development of Crohn's disease, and passive smoking exposure and maternal smoking at birth are associated with an increased risk of children developing IBD [29-30]. Hence exposure to environmental AhR agonists, such as PAH, through tobacco smoke or diet, may worsen the risk of developing IBD, while omega-3 fatty acid food supplementation may reduce it. These hypotheses need to be evaluated by *in vivo* experimental studies.

In conclusion we demonstrate in Caco-2 cells that PPARα activation by WY transcriptionally induces AhR expression through a PPRE site located within its promoter. This effect leads to a potentiation of CYP1A1 induction by 3MC, a potent AhR agonist. In addition to the modulation of xenobiotic metabolism, 3MC also

increases IL-1β and MMP9, which are major inflammatory targets. WY co-treatment abrogates the 3MC proinflammatory effect. Thus the anti-inflammatory effect of PPARα overrides the pro-inflammatory effect of AhR. Further experiments are required to elucidate the molecular events involved in these inflammatory events, and to evaluate *in vivo* the pro-inflammatory effect of AhR agonists such as PAH and their role in the development of IBD.

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Figure legends

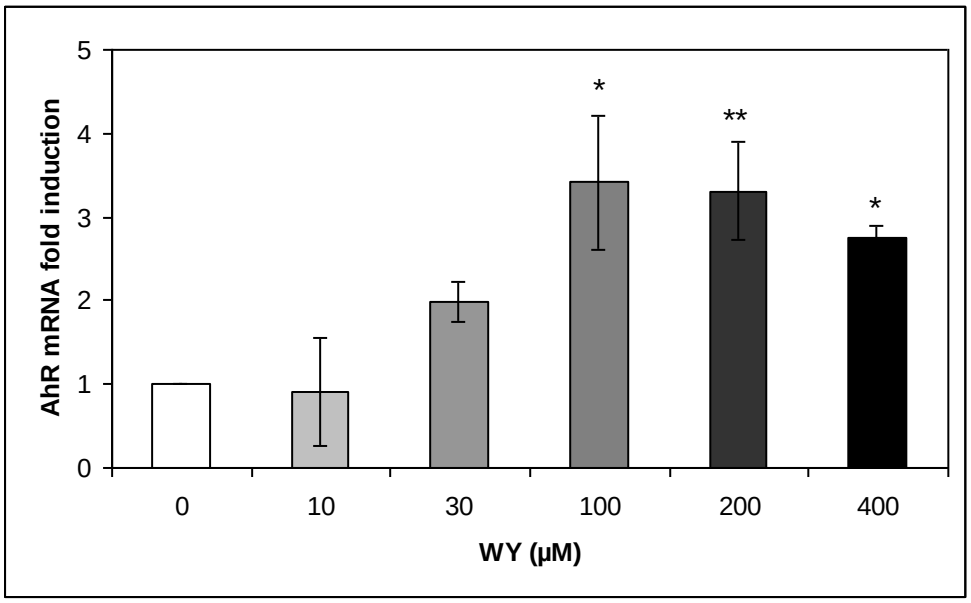
Fig. 1. WY induces AhR expression. A: Effect of increasing amounts of WY (0-400 µM) on AhR mRNA level in Caco-2 cells. *: $p < 0.01$, **: $p < 0.05$. B: Effect of WY (30 mg/kg) treatment on colic AhR mRNA level in wild type and PPARα knockout mice. Cwt: control wild type mice. WYwt: wild type mice treated with WY. Cko: control PPARα knockout mice. WYko: PPARα knockout mice treated with WY. *: $p < 0.01$.

Fig. 2. Effect of WY (200 µM) on AhR protein expression, in Caco-2 cells. A: untreated cells stained with DAPI. B: AhR immunostaining in untreated cells. C: cells treated with WY and stained with DAPI. D: AhR immunostaining in cells treated with WY.

Fig. 3. Effect of WY (200 µM) on AhR transcriptional activity. A: Effect of WY on AhR promoter activity in Caco-2 cells transfected with constructions harbouring the wild type sequence, or a sequence in which the PPRE site was mutated. B: Effect of WY on luciferase activity in Caco-2 cells transfected by a construction where the luciferase expression is under the control of the AhR PPRE. *: $p < 0.01$, **: $p < 0.05$.

Fig. 4. Effect of 3MC (1 µM), WY (200 µM), and 3MC+WY (1 and 200 µM respectively) on the mRNA of some inflammation target proteins, in Caco-2 cells. C: control cells. 3MC: cells treated with 3MC. WY: cells treated with WY. 3MC+WY: cells treated with 3MC and WY. *: $p < 0.01$.

A


AhR mRNA fold induction
5
4
3
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1
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10
30
100
200
400
WY (μM)

B

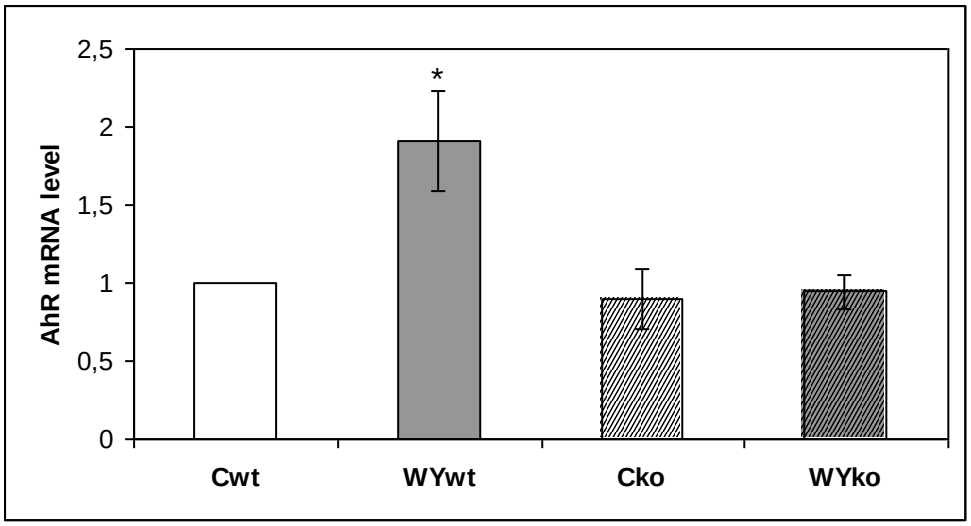

AhR mRNA level
2,5
2
1,5
1
0,5
0
*
Cwt
WYwt
Cko
WYko

Fig. 1

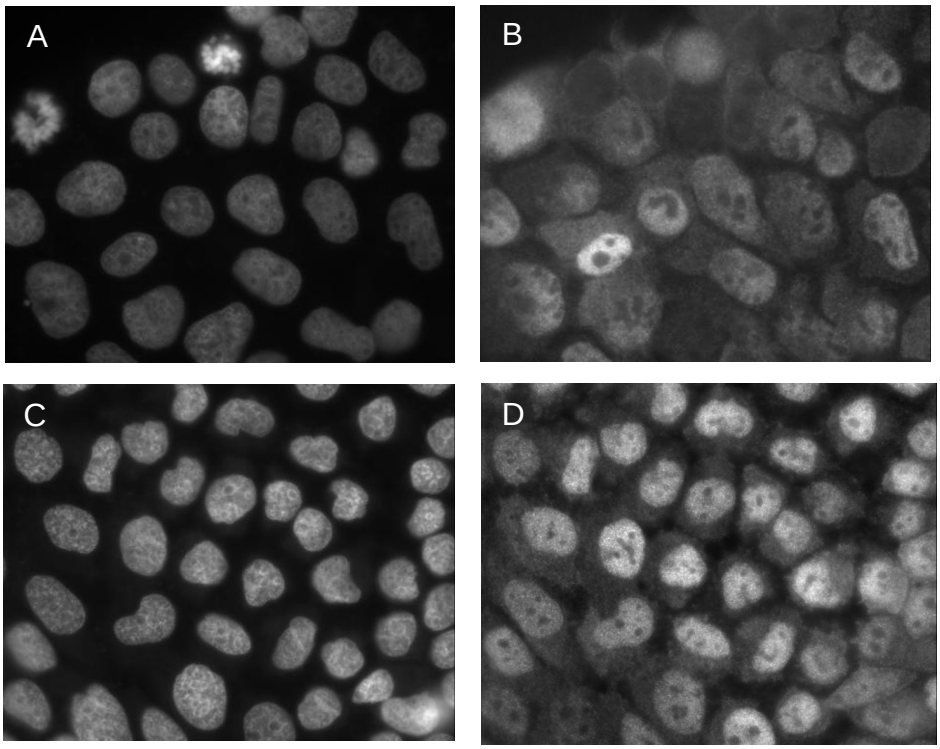
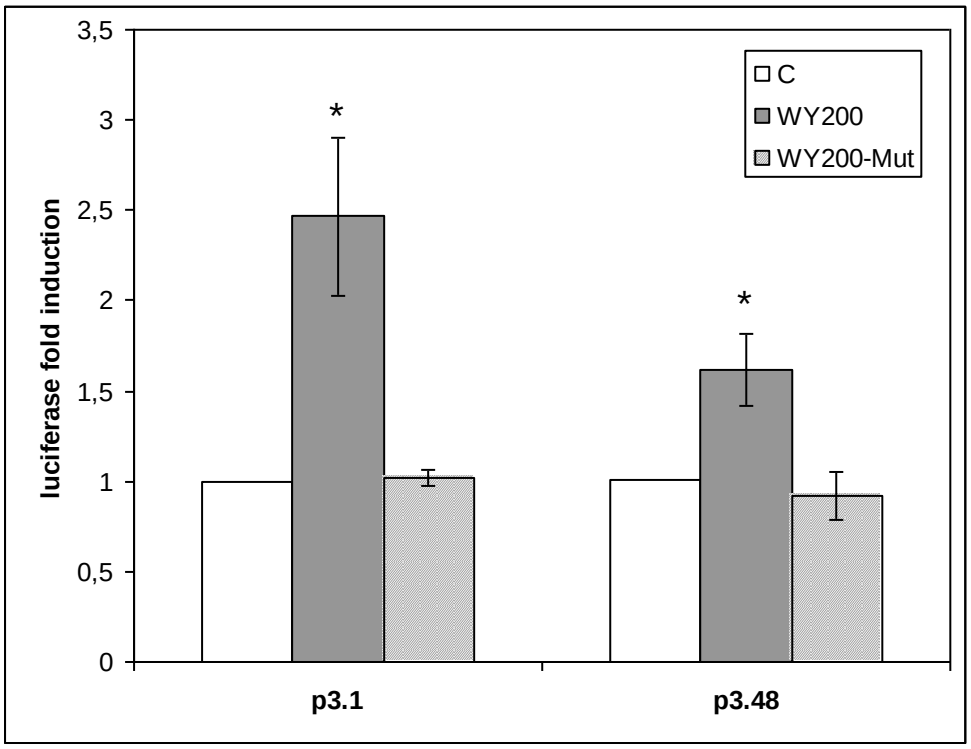


Fig. 2

A


3,5
3
2,5
2
1,5
1
0,5
0
luciferase fold induction
C
WY200
WY200-Mut
*
*
p3.1
p3.48

B

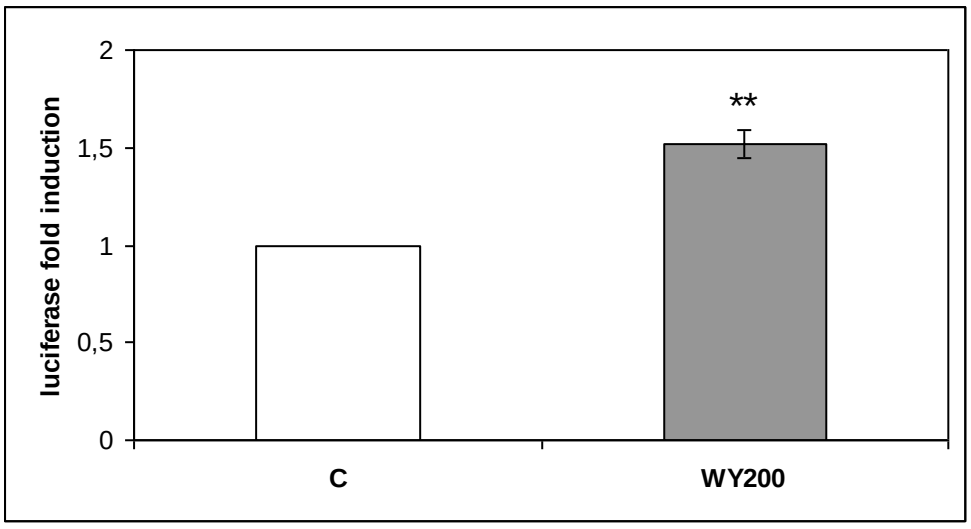

2
1,5
1
0,5
0
luciferase fold induction
**
C
WY200

Fig. 3

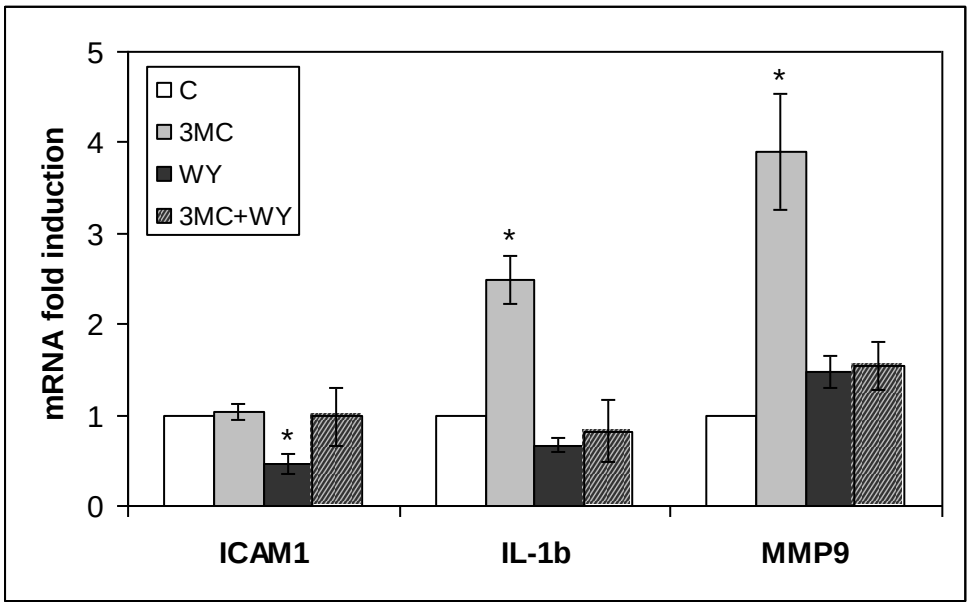


Fig. 4