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Detection of a functional xenobiotic response element in a widely employed FoxO-responsive reporter construct

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ABSTRACT

FHRE-Luc is a promoter reporter construct that is widely used to assess the activity of FoxO (forkhead box, class O) transcription factors. We here demonstrate that this promoter construct responds to exposure of HepG2 human hepatoma cells to known agonists of the aryl hydrocarbon receptor (AhR), 3-methylcholanthrene, benzo(*a*)pyrene, and 6-formylindolo[3,2-*b*]carbazole. However, FHRE-Luc activation did not coincide with FoxO DNA binding or changes in Akt-induced FoxO phosphorylation after treatment with AhR agonists. Testing FHRE-Luc deletion constructs and using AhR-deficient cells, we found that FHRE-Luc activation by AhR agonists is due to a functional xenobiotic-response element (XRE) spanning the backbone/insert border of the reporter plasmid. In conclusion, care must be taken when using FHRE-Luc to assess FoxO activity in response to stimuli that potentially interfere with xenobiotic signaling.

Introduction

Transcription factors of the FoxO (forkhead box, class O)[2] family are involved in the regulation of a broad variety of cellular processes, ranging from energy metabolism and glucose homeostasis to stress response and antioxidant defense [1]. Major levels of regulation of FoxO activity are posttranslational modification (most notably phosphorylation, acetylation and ubiquitination) as well as subcellular localization [2]. For instance, stimulation of cells with insulin results in insulin receptor-dependent stimulation of phosphoinositide 3′-kinase and the downstream kinase Akt, which in turn may phosphorylate FoxO transcription factors. Phosphorylation of FoxOs by Akt causes nuclear exclusion and inactivation of the transcription factors, resulting in an attenuation of expression of FoxO-regulated genes [1].

Based on these properties of FoxO transcription factors, several screening methods have been established to assess activity of the FoxO system, including the analysis of FoxO phosphorylation (e.g., by Western blotting), of FoxO subcellular localization (e.g., by immunohistochemistry), of FoxO DNA binding (e.g., by DNA fragment electrophoretic mobility shift analysis) or of the activity of FoxO-responsive promoter constructs in reporter gene assays.

Similar to FoxO transcription factors, the aryl hydrocarbon receptor (AhR) is stimulated by exogenous stimuli, including xenobiotics such as polycyclic aromatic hydrocarbons (PAHs), which are common environmental contaminants found in air, water and in food. The mode of activation of the AhR and of stimulation of its transcriptional activity drastically differs from that of FoxO transcription factors: whereas several more or less specific modes of stimulation of signaling cascades that entail a modulation of FoxO signaling were described (for an overview, see [3]), AhR is stimulated by direct binding of a suitable xenobiotic compound, leading to the release of AhR from cytoplasmic binding partners and its migration into the nucleus, where – upon dimerization with a transcriptional coregulator such as the AhR nuclear translocator (ARNT) – its binding to target sequences harboring the xenobiotic-response element (XRE) results in modulation of transcription (see [4] for a recent review).

We here demonstrate that data resulting from the use of a widely employed FoxO-responsive promoter construct, FHRE-Luc [5], may easily be misinterpreted, as the construct unexpectedly harbors a functional response element for AhR.

Despite the above-mentioned differences in stimulation of these signaling cascades, both FoxOs [6] and the AhR [4] share their being responsive to exogenous stimuli and exposure of cells to xenobiotics. As an overlapping pattern of response to cell stimulation may

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[2] *Abbreviations used:* AhR, aryl hydrocarbon receptor; PAH, polycyclic aromatic hydrocarbon; FHRE, forkhead responsive element; FoxO, forkhead box, class O; FICZ, formylindolo[3,2-*b*]carbazole; ARNT, AhR nuclear translocator; XRE, xenobiotic-response element; DMEM, Dulbecco's modified Eagle's medium; ATCC, American Type Culture Collection.

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therefore be expected, care must be taken to complement FHRE-Luc-based experiments with proper controls and tests for other aspects of FoxO function, if conclusions are to be drawn with respect to FoxO activity.

Materials and methods

Reagents and plasmids

All chemicals and enzymes were from Sigma (Steinheim, Germany), Merck (Darmstadt, Germany) or New England Biolabs (Ipswich, MA, USA), if not mentioned otherwise. 6-Formylindolo [3,2-*b*]carbazole was a kind gift from Dr. Ellen Fritsche, IUF, Düsseldorf. The FoxO1a expression plasmids [7,8] were a kind gift by Dr. Dieter Schmoll (Sanofi-Aventis, Frankfurt, Germany) or subclones of a BamHI/XhoI fragment of a FoxO1a expression plasmid (pSG5L HA FKHR wt; Addgene plasmid 10693) cloned into the pcDNA6.2/nLumio-DEST vector (Invitrogen) via pENTR3c using LR-Clonase (Invitrogen). The FoxO3a expression plasmid (HA-FoxO3a WT; Addgene plasmid 1787) as well as the FHRE-Luc construct (Addgene plasmid 1789 [5]) were provided by Dr. M. Greenberg (Children's Hospital, Boston, MA, USA) through Addgene (Cambridge, MA, USA). The pGL3-Promoter vector (pGL3-Prom) was from Promega (Madison, WI, USA). The FoxO4 expression plasmid was generated by subcloning a SalI/NotI fragment from a FoxO4 (AFX) expression plasmid kindly provided by Prof. B. M. T. Burgering (UMC Utrecht, The Netherlands) [9] into the pcDNA6.2/nLumio-DEST vector (see above). The XRE-driven luciferase plasmid (pGud-Luc6.1, termed "XRE-Luc" here) was generously provided by Dr. M. S. Denison (University of California, Davies, CA, USA).

FHRE-Luc$^{\Delta XRE}$ and FHRE-Luc$^{\Delta FHRE}$ were generated from FHRE-Luc by endonucleolytic digestion using MluI/PflF1 and MluI/BglII, respectively, producing blunt ends, filled with DNA polymerase I large fragment and religated using T4 ligase (Roche). FHRE-luc$^{\Delta FHRE/\Delta IRE}$ resulted from digestion of FHRE-Luc$^{\Delta FHRE}$ with KpnI and NotI and further digestion with mung bean nuclease to remove sticky ends. Sequences of plasmids were verified by DNA sequencing using the GL2 and RV3 primers.

Cell culture

HepG2 human hepatoma cells were used from two batches, one a kind gift of Dr. Johannes Bode (Heinrich-Heine-Universität Düsseldorf), the other purchased from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany), and were held in Dulbecco's modified Eagle's medium (DMEM, with

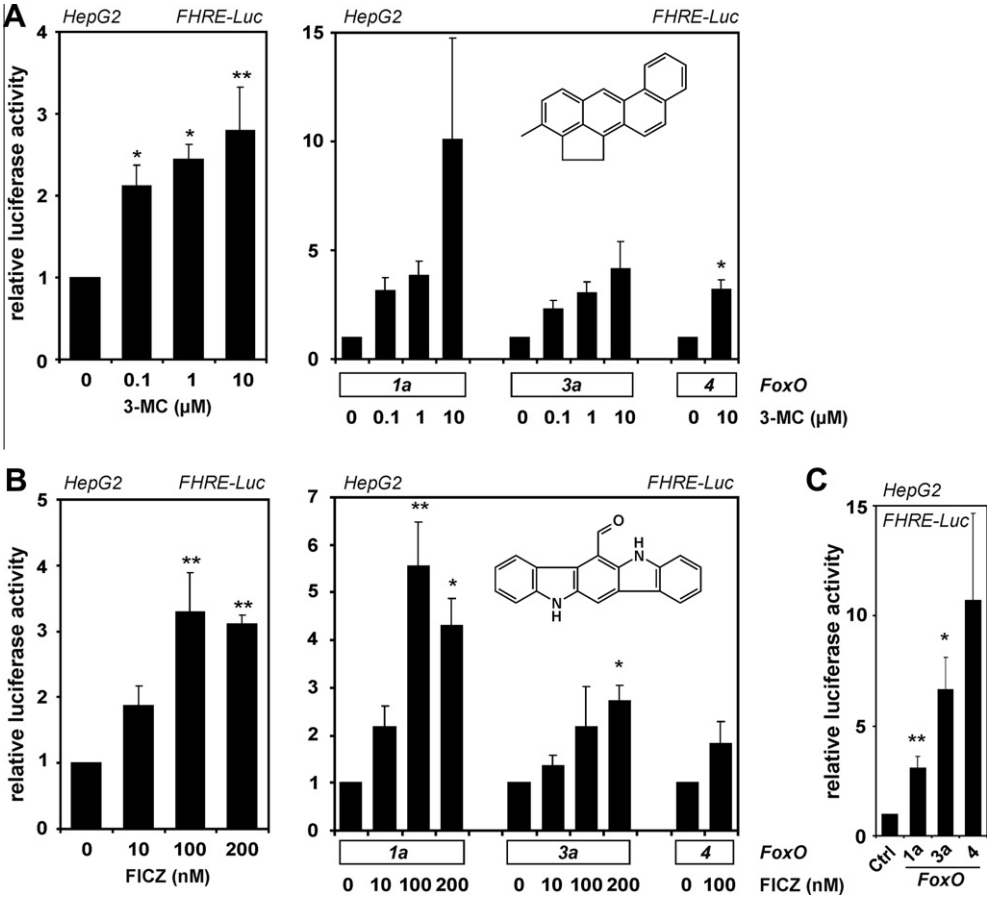

Fig. 1. Modulation of the FoxO-responsive promoter construct FHRE-Luc by arylhydrocarbon receptor agonists. (A) HepG2 cells were transfected with a promoter-luciferase reporter gene construct carrying FoxO-responsive elements (FHRE-Luc) and cotransfected with an expression plasmid control (pCI-NeO, left panel) or human FoxO1a, -3a or -4 expression plasmids (right panel). Twenty-four hours later, cells were exposed to 3-methylcholanthrene (3-MC) at the given concentrations or DMSO (0.1%, as solvent control at 0 μM 3-MC) in serum-free medium for another 18 h, followed by analysis of luciferase activity in cell lysates. Luciferase activities in cells overexpressing a given FoxO isoform were set to 1 and changes in the presence of 3-MC related to it (right panel). Data are given as means of three to ten independent experiments ± SEM. (B) HepG2 cells were transfected with FHRE-Luc and control or expression plasmids for FoxO1a, -3a or -4 as in (A), followed by treatment with 6-formylindolo[3,2-*b*]carbazole (FICZ), in the given concentrations or DMSO. Data are given as means of three to fourteen independent experiments ± SEM. (C) Luciferase activities in cells transfected with FHRE-Luc and overexpressing the respective human FoxO isoform relative to cells cotransfected with a control plasmid (Ctrl). Data are given as means of three to eight independent experiments ± SD.

4.5 g/l glucose, PAA, Pasching, Austria) supplemented with 10% (v/v) fetal calf serum (PAA), 2 mM glutamax (Invitrogen, Karlsuhe, Germany), non-essential amino acids (PAA) and penicillin/streptomycin (PAA), at 37 °C in a humidified atmosphere with 5% (v/v) CO_2. Hepa1c1-c7 (ATCC# CRL-2026), Hepa1c1-c12 (ATCC# CRL-2710) and Hepa1c1-c4 (ATCC# CRL-2717) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA) and were cultured like HepG2 cells, but without the addition of non-essential amino acids to DMEM.

Western blotting

For analysis of Akt and FoxO phosphorylations, cells were lysed in 2× SDS–PAGE buffer (125 mM Tris/HCl, 4% (w/v) SDS, 20% glycerol, 100 mM dithiothreitol and 0.02 (w/v) bromophenol blue, pH 6.8) after treatment, followed by brief sonication. Samples were applied to SDS–polyacrylamide gels of 10% (w/v) acrylamide, followed by electrophoresis and blotting. Immunodetection was performed using the following antibodies: anti-phospho-Akt (S473) and anti-phospho-FoxO1a/FoxO3a (T24/T32) were from Cell Signaling Technology (Danvers, MA), anti-GAPDH was from Millipore (Billerica, MA); horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and anti-mouse IgG secondary antibodies were from Dianova (Hamburg, Germany) and Amersham (Munich, Germany), respectively.

For phospho-FoxO1a detection, antibody incubation was performed in 5% (w/v) BSA in Tris-buffered saline containing 0.1% (v/v) Tween-20 (TBST); for analysis of all other antibodies, incubations were in 5% (w/v) non-fat dry milk in TBST.

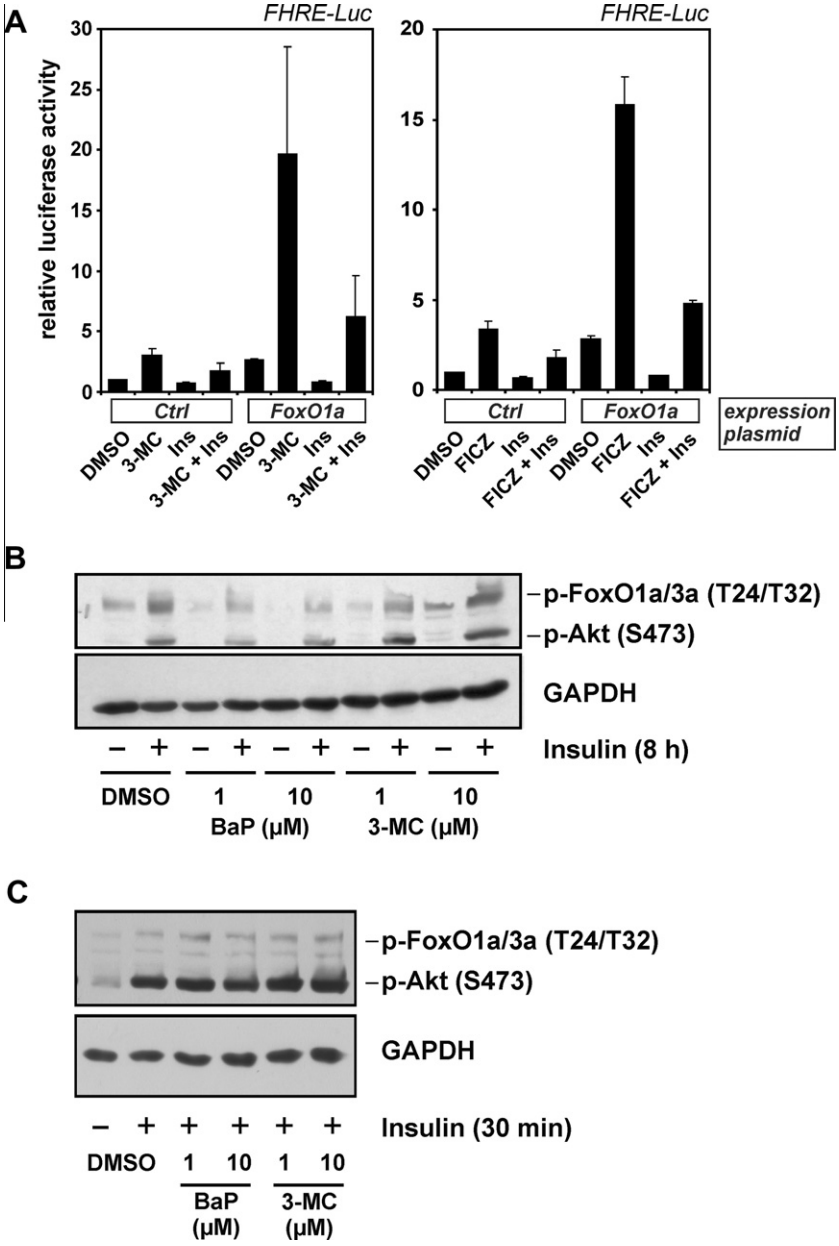

Fig. 2. Insulin signaling and AhR agonists. (A) HepG2 cells were transfected with FHRE-Luc and a control or FoxO1a expression plasmid as in Fig. 1. Twenty-four hours later, cells were exposed to 3-MC (10 μM, left panel), FICZ (100 nM, right panel), DMSO (0.1%) and/or insulin (100 nM) in serum-free medium for another 18 h, followed by analysis of luciferase activity in cell lysates. Data are given as means of three independent experiments ± SEM. (B and C) HepG2 cells were held in serum-free medium for at least 18 h prior to exposure to 3-MC or B(a)P in the absence or presence of insulin (100 nM) for 8 h (B). Alternatively, exposure was for 8 h, with insulin added only for the last 30 min (C). DMSO (0.1%) was used as solvent control. Analysis of FoxO and Akt phosphorylation in cells was done by Western blotting employing phosphospecific antibodies. Glyceraldehyde 3-phosphate dehydrogenase levels were analyzed as loading controls. The blots shown are representative of three independent experiments with similar results.

Luciferase reporter gene assays

Cells were grown to approximately 60% confluence in 9 cm^2 cell culture dishes and transfected using Polyfect reagent (Qiagen, Hilden, Germany). Luciferase promoter constructs (0.75 μg) were cotransfected with 0.1 μg of renilla luciferase control plasmid (pRL-SV40, Promega, Mannheim, Germany) and either 0.75 μg of FoxO expression plasmids or control plasmids (pCI-Neo). All transfections were performed in serum-free medium (containing glutamax, penicillin/streptomycin and non-essential amino acids). Exposure of cells to PAHs or insulin commenced 24 h after transfection. For the respective treatment, cells were washed once with PBS and then held in serum-free medium containing 3-methylcholanthrene (3-MC, 100 nM, 1 μM or 10 μM), benzo(*a*)pyrene (B(a)P, 100 nM, 1 μM or 10 μM), 6-formylindolo[3,2-*b*]carbazole (FICZ, 10 nM, 100 or 200 nM) and/or insulin (100 nM) for 18 h prior to determination of luciferase activities using the Dual Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Luciferase assays in Figs. 5 and 6A were performed as described previously [10]: cells were grown in 24-well plates and promoter constructs (1.6 μg) were cotransfected with 0.1 μg of renilla luciferase control plasmid (pRL-SV40, Promega, Mannheim, Germany) and 1.6 μg of FoxO expression plasmids as indicated. Transfections were performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Exposure of cells to 3-MC commenced 18 h after transfection. For treatment, cells were washed once with PBS and then held in serum-free medium containing 3-methylcholanthrene for 24 h prior to determination of luciferase activities using the Dual Luciferase Reporter Assay System (Promega). AhR knockdown by siRNA (IDT, Coralville, IA, USA) and control siRNA treatment (Silencer Select Negative Control No. 2, Applied Biosystems, Foster City, CA, USA) were performed as described previously [10].

FoxO DNA binding

FoxO DNA binding activity was assayed employing an ELISA-based FoxO DNA binding assay (TransAM FKHR, Active Motif, Rixensart, Belgium) according to the manufacturer's instructions. HepG2 cells were grown to approximately 80% confluence in 79 cm^2 cell culture dishes. Cells were held in serum-free medium for 24 h prior to exposure to 10 μM 3-MC, 100 nM FICZ or DMSO (0.1%) with or without insulin (100 nM) in serum-free medium for 2 h. Cells were harvested and nuclear protein extracted. Nuclear extracts were applied to 96-well plates coated with oligonucleotides containing FoxO DNA binding elements. Bound active FoxO was then detected using an antibody directed against FoxO1a, the binding of which was assayed employing a secondary antibody conjugated with HRP.

Statistical analysis

Analysis of statistical significance was done by paired Student's *t*-test with $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ as levels of significance.

Results and discussion

Stimulation of FHRE-Luc promoter activity by AhR agonists

As FoxO transcription factor activities are modulated not only by insulin and other growth factors [1] but also by stressful and environmental stimuli, such as reactive oxygen species, ROS [9], heavy metal ions [6,11,12] or polyphenols [13], we hypothesized that an interaction might occur between FoxOs and the known xenobiotic-responsive AhR transcriptional regulatory system. To test this hypothesis, we transiently transfected HepG2 human hepatoma cells with a widely employed promoter/luciferase reporter construct carrying three FoxO binding sites originally derived from the human Fas ligand promoter [5]. In addition to the FHRE-Luc reporter, cells were also in part transfected with expression plasmids for the ubiquitously expressed FoxO isoforms 1a, 3a and 4 in order to analyze the cellular response in the presence of abundant FoxO protein.

Following transfection, cells were exposed for 18 h to known agonists of the AhR, the polycyclic aromatic hydrocarbons 3-methylcholanthrene (3-MC) and benzo(*a*)pyrene, B(a)P [14], as well as the tryptophan derivative, 6-formylindolo[3,2-*b*]carbazole (FICZ) [15–17]. Promoter activity was then assessed by analysis of luciferase activity and was found to be significantly elevated by up to 3-fold over control upon exposure to 3-MC (0.1–10 μM, Fig. 1A, left panel). This increase was even more pronounced after overexpression of any of the three tested FoxO isoforms (Fig. 1A, right panel).

Similar to 3-MC, B(a)P enhanced FHRE-Luc promoter activity, with 0.1 μM of B(a)P causing a 50% increase (data not shown). Stimulation of transfected cells with FICZ was also most pronounced at these low concentrations (Fig. 1B, left panel) and was even further elevated in the presence of overexpressed FoxO1a (Fig. 1B, right panel). Overexpressed FoxO isoforms were functional, as they strongly enhanced FHRE-Luc activity *per se* (Fig. 1C).

Transcriptional activity of FoxO proteins is physiologically controlled by insulin. In order to test whether the effects observed with FHRE-Luc were due to an interference of AhR agonists with insulin signaling towards Akt and FoxO, we exposed HepG2 cells transiently transfected with FHRE-luc and a control or a FoxO1a expression plasmid to both insulin and/or AhR agonists (Fig. 2A). As expected, FoxO1a overexpression elevated FHRE-luc activity, while insulin (100 nM) treatment resulted in a reduction of

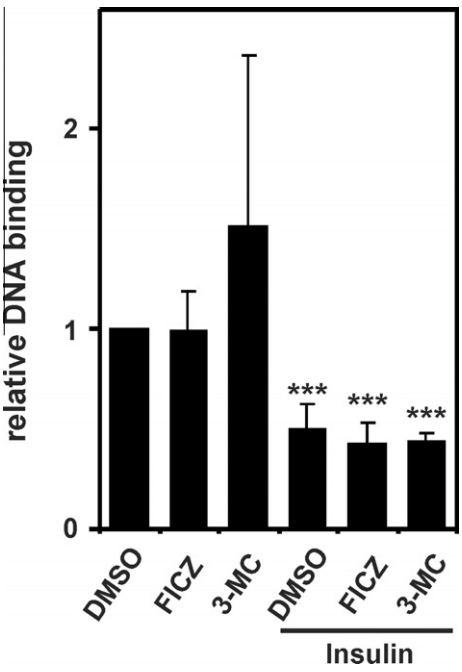

Fig. 3. Modulation of FoxO-DNA binding after stimulation with 3-methylcholanthrene or FICZ. HepG2 cells were held in serum-free medium for 24 h prior to exposure for 2 h to 3-MC (10 μM), FICZ (100 nM) or DMSO (0.1%) in serum-free medium. Insulin (100 nM) was present where indicated. Nuclear extracts were prepared and FoxO binding to target DNA determined using an ELISA-based FoxO DNA binding assay. Data are given as means of five independent experiments ± SD; ***Significantly different from DMSO control.

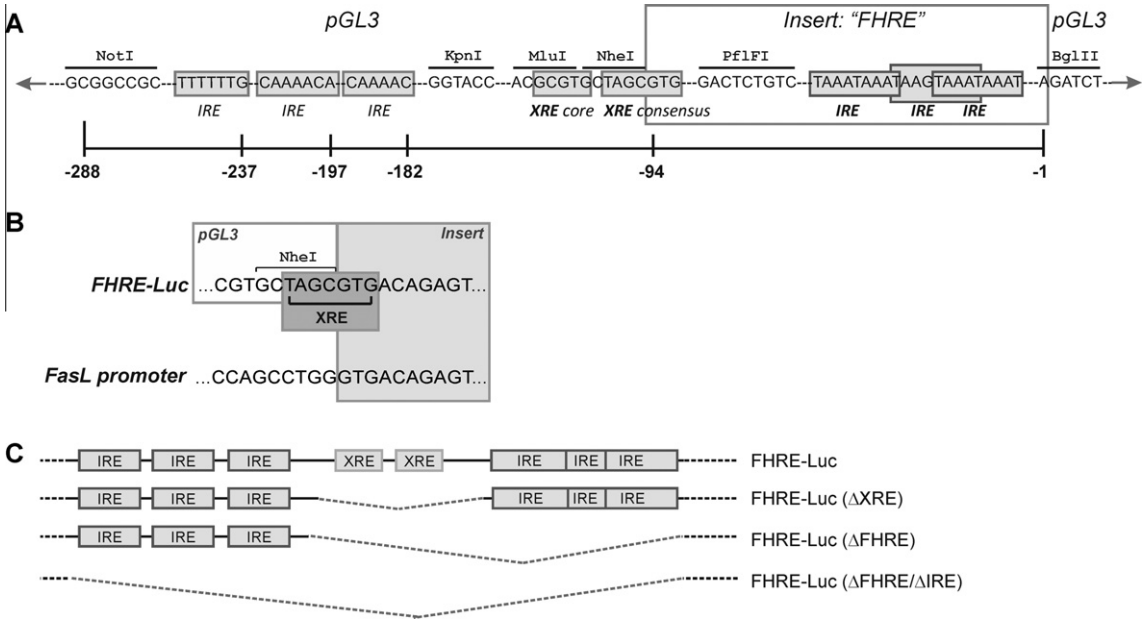

Fig. 4. FHRE-Luc sequence elements and deletion constructs. (A) Schematic representation of transcription factor binding sites or potential binding sites and their localization in FHRE-Luc. Forkhead-responsive elements, FHRE, are also termed insulin-responsive elements, IRE, here. XRE, xenobiotic-responsive element. (B) Comparison of FHRE-Luc and the Fas ligand (FasL) promoter sequence (GenBank ID: AF035584.1) [20] that the FHRE-Luc insert was derived from [5]. (C) Schematic representation of deletion constructs derived from FHRE-Luc.

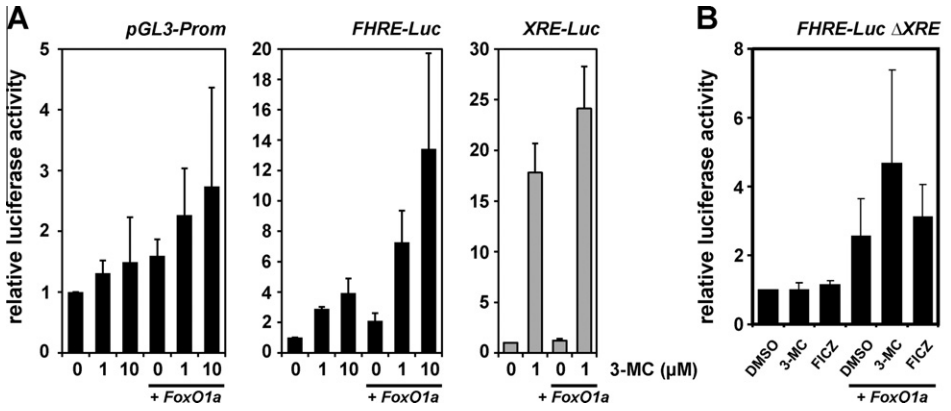

Fig. 5. Role of AhR in modulation of FHRE-Luc by AhR agonists. (A) HepG2 cells were transfected with empty reporter plasmid (pGL3-Prom), FHRE-Luc or an XRE-driven reporter (XRE-Luc), as well as FoxO1a expression plasmid where noted. 18 h later, cells were exposed to 3-MC in serum-free medium for another 24 h, followed by analysis of luciferase activity in cell lysates. Data are given as means of three (XRE-Luc: two) independent experiments performed in quadruplicates + SD (XRE-Luc: error bars denote difference between average and max value). (B) HepG2 cells were transfected with FHRE-Luc$^{\Delta XRE}$ and a control or FoxO1a expression plasmid. Twenty-four hours later, cells were exposed to 3-MC (10 μM), FICZ (100 nM) or DMSO (0.1%) in serum-free medium for another 18 h, followed by analysis of luciferase activity in cell lysates. Data are given as means of three to eight independent experiments ± SD.

luciferase activity, both with and without FoxO1a overexpression. As before, exposure to 3-MC (Fig. 2A, left) and FICZ (Fig. 2A, right) induced an increase in promoter activity. Interestingly, coincubation of 3-MC or FICZ with insulin resulted in promoter activities close to control levels, suggesting that both the lowering effect of insulin and the enhancing effect of AhR agonists simply added up. This would suggest that insulin and AhR agonists either antagonistically affect the insulin signaling cascade to FoxOs or that they affect FHRE-Luc in two independent ways.

To further investigate whether insulin signaling to FoxOs is modulated by AhR agonists, we treated cells with insulin in the absence or presence of 3-MC or B(a)P, followed by analysis of Akt and FoxO phosphorylation by Western blotting. Employing a mixture of phospho-specific antibodies detecting Ser-473 phosphorylation of Akt and Thr-24 phosphorylation of FoxO1a (or Thr-32 in FoxO3a), we found no significant change in insulin-induced Akt and FoxO phosphorylation in the presence of 3-MC or B(a)P (Fig. 2B and C).

Detection of a functional XRE in FHRE-Luc

In order to verify stimulation of FoxO activity by AhR agonists that was suggested by the FHRE-Luc experiments, we tested whether treatment of human hepatoma cells with AhR agonists leads to enhanced binding of FoxO proteins to FoxO response elements. We performed an ELISA-based FoxO DNA binding assay with nuclear extracts of HepG2 cells exposed to DMSO (solvent control), 3-MC (10 μM) or FICZ (100 nM) in the absence or presence of insulin (100 nM). As expected, insulin clearly downregulated FoxO DNA binding, but interestingly none of the AhR agonists did (Fig. 3). Moreover, and different from the results

obtained in FHRE-Luc-based experiments, the insulin effect was not antagonized by AhR agonists.

This discrepancy prompted us to look for AhR ligand-responsive elements (xenobiotic-response elements, XRE) in the FHRE-Luc nucleotide sequence. We indeed noted an element matching the consensus sequence for the most prominent active form of AhR, the AhR/AhR nuclear translocator (ARNT) heterodimer, 5′-TNGCGTG-3′ [18,19]. Interestingly, this sequence element spans the border between pGL3 plasmid backbone and the 5′-end of the FHRE insert (Fig. 4A). When comparing FHRE-Luc with the sequence of the 5′-untranslated region of the human Fas ligand [20] that the FHRE insert was derived from [5], the 5′-TNGCGTG-3′ putative XRE was not found in the corresponding Fas ligand promoter region (Fig. 4B), suggesting that the potential XRE was generated during creation of FHRE-Luc.

In order to test whether this sequence represents a functional XRE, we (i) compared the modulation of the empty pGL3-Prom plasmid with that of FHRE-Luc and (ii) created a deletion construct, FHRE-LucΔ^{XRE} (Fig. 4C), lacking this putative XRE as well as another adjacent 5′-GCGTG-3′ element found in the pGL3 plasmid background, and which represents a sequence that was recognized as the core recognition motif of an AhR/ARNT heterodimer previously [18,19].

As demonstrated in Fig. 5A, 3-MC induces only minute changes in luciferase activity in cells transfected with the empty pGL3-Promoter plasmid – both with and without cotransfection of FoxO1a – whereas both FHRE-Luc and an AhR-responsive XRE-Luc plasmid respond as expected: 3-MC induces FHRE-Luc activity, and it strongly enhances XRE-Luc activity, whereas FoxO1a overexpression stimulates FHRE-Luc but not XRE-Luc.

The fact that the empty pGL3-Prom plasmid does not seem to be responsive to 3-MC exposure implies that the above-mentioned 5′-GCGTG-3′ XRE core element (see also Fig. 4A) does not contribute to the FHRE-Luc response to AhR agonists. In contrast, the

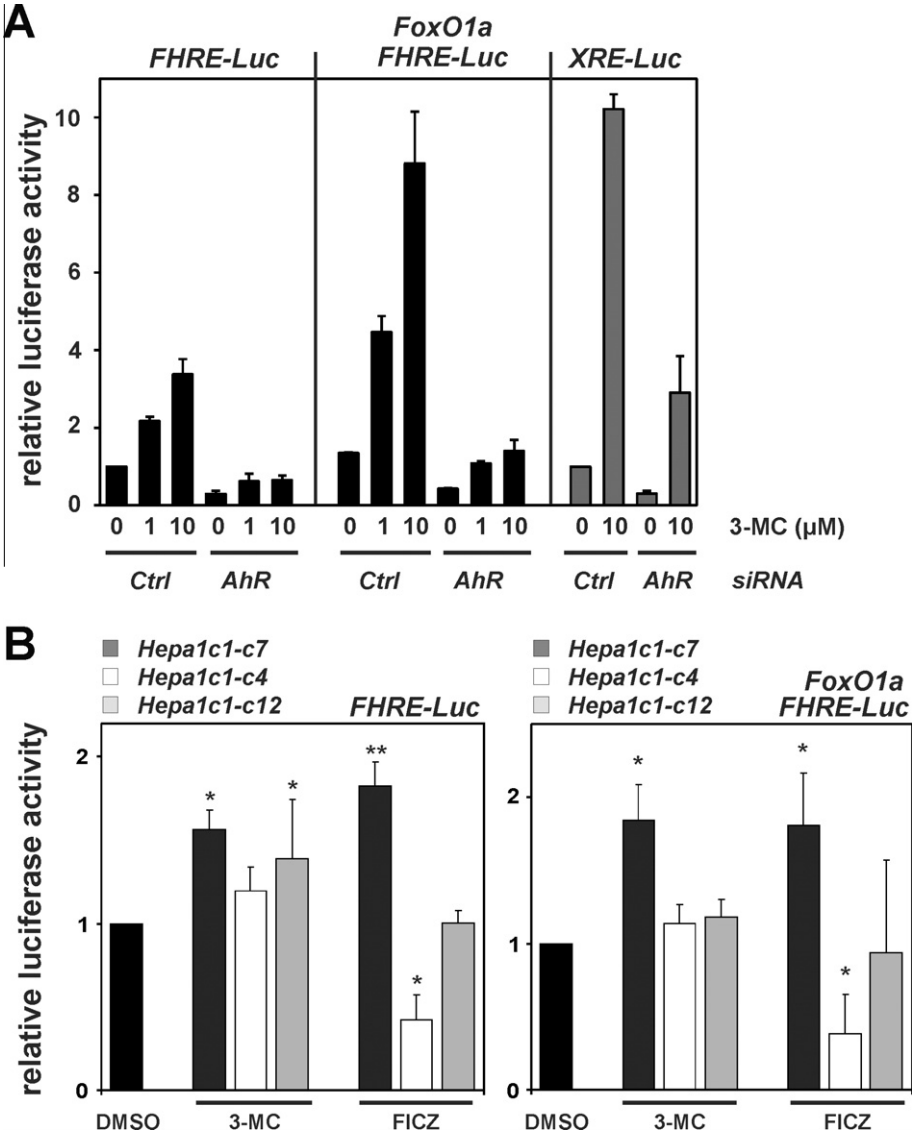

Fig. 6. Role of AhR in modulation of FHRE-Luc by AhR agonists. (A) HepG2 cells were transfected with control siRNA or AhR-specific siRNA, followed by transfection with FHRE-Luc or an XRE-driven reporter (XRE-Luc), as well as FoxO1a expression plasmid where noted. 18 h later, cells were exposed to 3-MC in serum-free medium for another 24 h, followed by analysis of luciferase activity in cell lysates. Data are given as means of two independent experiments performed in quadruplicates. Error bars denote the difference between average and max value. (B) Modulation of FHRE-Luc promoter activity by 3-MC or FICZ in murine hepatoma cells. Hepa1c1c-7, -c4 or -c12 cells were transfected with FHRE-Luc and cotransfected with a control expression plasmid (pCI-Neo, left panel) or a FoxO1a expression plasmid (right panel). Twenty-four hours later, cells were exposed to 3-MC (10 μM), FICZ (100 nM) or DMSO (0.1%, as solvent control) in serum-free medium for another 18 h, followed by analysis of luciferase activity in cell lysates. Luciferase activities under control conditions (DMSO as solvent control) in any of the cell types were set to 1 and changes in the presence of 3-MC or FICZ related to it. Data are given as means of four independent experiments ± SEM (left panel) or SD (right panel).

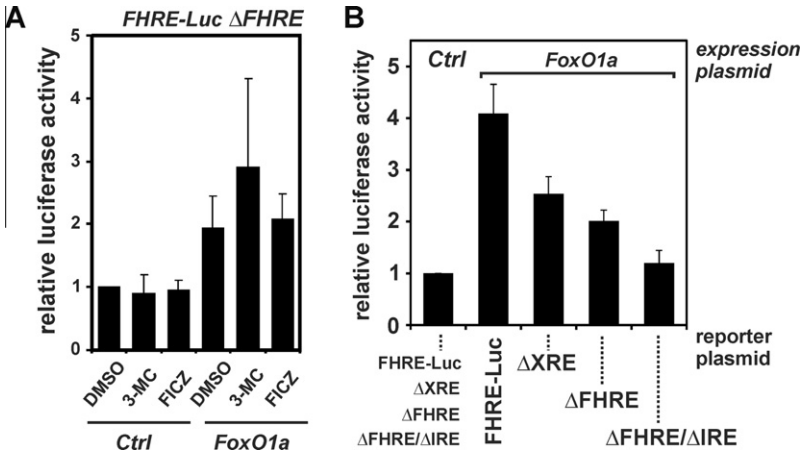

Fig. 7. FHRE-Luc and deletion constructs: analysis of responsiveness to FoxO1a overexpression. (A) HepG2 cells were transfected with FHRE-Luc$^{\Delta FHRE}$ plasmid and a control or FoxO1a expression plasmid. Twenty-four hours later, cells were exposed to 3-MC (10 μM), FICZ (100 nM) or DMSO (0.1%) in serum-free medium for another 18 h, followed by analysis of luciferase activity in cell lysates. Data are given as means of three to eight independent experiments ± SD. (B) HepG2 cells were transiently transfected with a control or FoxO1a expression plasmid as well as with a FHRE-Luc, FHRE-Luc$^{\Delta XRE}$, FHRE-Luc$^{\Delta FHRE}$ or FHRE-Luc$^{\Delta FHRE/\Delta IRE}$ reporter plasmid. Luciferase activities in cells carrying control expression plasmid (Ctrl) were set to 1, irrespective of the transfected reporter plasmid. Data are given as means of three to nine independent experiments ± SEM.

5′-TNGCGTG-3′ XRE consensus sequence at the pGL3 plasmid backbone/FHRE insert border appears to be essential: FHRE-LucΔ^{XRE} was no longer responsive to 3-MC or FICZ treatment both with and without overexpressed FoxO1a (Fig. 5B). As expected, FoxO overexpression caused an elevation of promoter activity, as the FoxO-responsive sites are still present in FHRE-LucΔ^{XRE}. Interestingly, FoxO-induced elevation of promoter activity was much less pronounced in FHRE-LucΔ^{XRE} than in FHRE-Luc (see also below, Fig. 7B), pointing to an important role of the XRE region for FoxO-induced promoter activation.

In order to further test whether FHRE-Luc contains a functional XRE, we lowered AhR levels in HepG2 cells by treatment with an AhR-specific siRNA prior to exposure to 3-MC. In cells treated with AhR-specific siRNA, basal FHRE-Luc activity was lowered to approximately 1/3 of its activity in control siRNA-treated cells – both in the absence and in the presence of cotransfected FoxO1a expression plasmid (Fig. 6A). Similarly, the induction of FHRE-Luc activity by 3-MC was strongly attenuated in cells treated with AhR-specific siRNA (Fig. 6A). As with FHRE-Luc, both basal and 3-MC-induced activity of XRE-Luc were lowered to approximately 1/3 in cells treated with AhR-specific siRNA, implying that AhR was successfully, albeit only partially, depleted (Fig. 6A).

To corroborate these data, we also transfected Hepa1c1-c7 murine hepatoma cells as well as ARNT-deficient (Hepa1c1-c4) and AhR-deficient (Hepa1c1-c12) clones [21,22] with FHRE-Luc (Fig. 6B, left panel) or FHRE-Luc plus a FoxO1a expression plasmid (Fig. 6B, right panel), followed by exposure to 3-MC (10 μM) or FICZ (100 nM) for 18 h. As with HepG2 human hepatoma cells, an increased promoter activity after stimulation with 3-MC (10 μM) or FICZ (100 nM) both under basal conditions and with FoxO1a overexpression was observed in murine Hepa1c1-c7 cells (Fig. 6B). In contrast, only a minor or no increase of FHRE-luc activity after exposure to 3-MC or FICZ was observed in cells carrying an ARNT-(Hepa1c1-c4) or AhR-(Hepa1c1-c12) mutation (Fig. 6B), suggesting that either of the proteins was required for full FHRE-Luc activation by AhR agonists.

Interestingly, the response to FICZ was different from that to 3-MC in Hepa1c1-c4 cells in that a downregulation, rather than the mere loss, of FHRE-Luc activity was induced by FICZ in the absence of functional ARNT (Fig. 6B). While this finding may point to a functional relation between FoxOs and ARNT, a simple interaction between these two would not explain the difference between 3-MC and FICZ. Therefore, the reasons for, and implications of, this finding remain to be characterized.

Further analysis of FHRE-Luc

As noted above (Fig. 5B), FoxO overexpression caused an elevation of FHRE-LucΔ^{XRE} promoter activity, which was expected because the FoxO-responsive IREs are still present in that construct. To control for the relevance of these IREs in mediating the response to FoxO1a overexpression, we further deleted the FHRE insert, including these IREs. To our surprise, the resulting FHRE-LucΔ^{FHRE} (see Fig. 4C) still responded to FoxO overexpression, which doubled luciferase activity (Fig. 7A). Analysis of the pGL3 plasmid in a region proximal to the insert resulted in identification of three potential IRE sequences approximately 200 bp upstream of the insert (see Fig. 4A). We then deleted these IREs to test whether FoxO responsiveness of the reporter could be abrogated. The resulting reporter deletion construct (FHRE-Luc$\Delta^{FHRE/\Delta IRE}$, see Fig. 4C) indeed no longer responded to FoxO1a overexpression in HepG2 cells (Fig. 7B), indicating that three IREs that are part of the pGL3 backbone may, to some extent, contribute to the stimulation of reporter gene expression upon FoxO1a overexpression.

Conclusions

FHRE-Luc is a reporter construct widely employed for the screening of FoxO transcription factor activities. We used this construct to test for an interaction between AhR and FoxO by screening for an activation of FHRE-Luc in cells exposed to AhR agonists but found that FHRE-Luc, in addition to the insulin-responsive elements that confer its FoxO-responsiveness, contains a functional xenobiotic-response element, XRE, that is responsible for the reporter plasmid's responsiveness towards AhR agonists. As both AhR and FoxO transcription factors may respond to similar sets of stimuli – such as flavonoids [13,23] or AhR agonists that happen to generate reactive oxygen species [24] which, in turn, would modulate FoxOs [3] – FHRE-Luc is suggested for use in assessment and screening of FoxO activities only if a role of the AhR can be excluded. We recommend to employ the XRE deletion construct for such FoxO activity screening rather than the full FHRE-Luc, if AhR stimulation cannot be excluded.

Different from reporter gene assays, neither Western analysis of Akt-dependent FoxO phosphorylation nor FoxO DNA binding analysis (Figs. 2 and 3) provided hints as to a direct or indirect interaction between AhR agonists and FoxO transcription factors. Interestingly, however, deletion of the XRE region from FHRE-Luc

also attenuated FoxO-induced promoter activation (Fig. 7B), pointing to a possible interaction between AhR and FoxO, the nature of which is yet to be identified.

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