内分泌攪乱化学物質の低用量作用と毒性学のあたらしい課題

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内分泌攪乱化学物質(いわゆる環境ホルモン)がヒトや野生生物の生殖に影響を与えるという危惧が新聞などで取り上げられてから10年以上が過ぎた．先頃は哺乳瓶に由来するビスフェノールAや玩具に関するフタル酸エステル類の扱いが取り上げられたが，ひと頃のように大きな話題にはならなかった．けだし内分泌攪乱問題は過去の出来事のように感じている人は少なくないかもしれない．しかしそうした人びとにも，その後この問題がどうなっているのか訝(いぶか)しく感じている向きはこれまた少なくないであろう．それだけに，内分泌攪乱化学物質問題とは，いったい何だったのか，いまそれはどんな意味をもって人々に関わっているのか．そんな点を中心に，これまでにわかってきたことを整理してみたいと思う．

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毒性学では，比較的高用量域における実験的生体反応の用量相関直線を，実験データ以下の低用量域へ外挿して無反応域[*1]を求め，それら無反応域よりも低い用量での生体影響を“無影響”と推定している．無反応域以下では，障害性はないと考えるわけである．平易な論理であるが，或る事柄が“ナイ”ということを予測し，保証するということは，科学の方法では大いに稀少なことである．必然的にこの論理には，用量相関が線形であるべきことなど様々の前提条件が求められ，これが破れるとその保証が効かなくなる．

しかし線形の用量相関を自明とする安易な認識とも相俟って，危惧された物質の環境中での曝露量は充分に微量と考えられたから，既知の無影響量以下の内分泌攪乱化学物質の曝露が，環境生物やヒトに生体影響を惹き起こす可能性は想定外であった．取り上げられた例もフロリダのアポプカ湖や五大湖での事故のように極端なケースにならざるを得なかった[*2]．だから低用量の内分泌攪乱化学物質にヒトや野生生物の生存を危うくする様々の危惧があるとして，これらが社会問題化したとき，環境生物やヒトで危惧される事象の事実関係もさることながら，もし事実とすれば“なぜ見落とされたのか”，あるいは，実験動物の観察でなぜ見いだされなかったのか，という疑問がまず取り上げられた．確かに，内分泌ホルモンに類する受容体を通じた低用量の影響が関与する可能

[*1] 無作用量(NOEL: no observed effect level)とか，無影響量(NOAEL: no observed adverse effect level)と呼ばれる．

[*2] たとえば，フロリダ州のアポプカ湖では，1980年，dicofolおよびDDTとその代謝物の汚染によりワニの棲息数が減少した．ミシガン湖周辺のカモメでは，DDT/DDEによるとされる雌化現象が観察された．

性などは，レイチェル・カーソンの『沈黙の春』[1]を思い起こした少なからぬ研究者が指摘した．経済協力開発機構(OECD)の試験開発部門が，従前，ホルモン剤の開発などに用いて知られていた“子宮腫大試験”を，環境中の化学物質の調査用にまったく新しく取り上げて調べ直すことになった経緯も，生体のホルモン受容体を介した影響への視点が働いていたし，世界保健機関(WHO)の化学物質安全計画部門がさらなる新たな試験法の開発の重要性を強調した所以でもあった．

こうした中で，高用量域から低用量域への直線外挿の如何を調べてゆくうちに，内分泌ホルモン影響には，それまで認識されていなかった「低用量作用」があることがわかってきた．たとえば，機構の異なるいくつかの複合影響をもたらす物質で生体影響について用量相関を見ると，しばしば非線形反応を呈し，低用量域ではじめて浮かび上がってくる性質が見いだされる．内分泌攪乱化学物質の低用量作用は，追試につぐ追試をうみ，にわかに注目を集めることとなったが，当時，メカニズムなど原理的な説明がつかなかったこととも相俟って，内分泌攪乱現象の真偽の決着はつかなかった[*3]．

かくして，内分泌攪乱化学物質に関する最初の国際ワークショップ，ウェイブリッジ会議から10年を経た2007年，その10周年のワークショップが，フィンランド科学アカデミーと欧州委員会(EU)の主催によりヘルシンキで開催された．そこでは，相加的で，無作用量(閾値)の認められない反応や，感染・免疫機構ないし神経・行動を中心に，これまで知られていなかった低用量影響がつぎつぎと紹介された．内分泌攪乱化学物質の生体影響の詳細には，依然として未知の要素が含まれているものの，ようやく，その片鱗が明らかになってきたのを感じた．

それらを取り入れてこれまでの経過を振り返れば，冒頭で述べた毒性学の論理の前提条件が，どこかで破れていたといわざるを得ない．しかもそれは，想定外の非線形反応にもとづく作用にとどまらず，未知の事柄を含む生物学の認識そのものに関わっていたため，条件の破綻として気付かれなかったものである．近代毒性学の中でいまだ理論化されていない論理の破れ，それは何だったのか．なぜ旧来の毒性学はこれを明らかにできなかったのか．いま低用量問題の本質のありかを解く意義は，ここにある．

「低用量問題」とは[*4]

内分泌攪乱化学物質の性質として，低用量作用が取り上げられたのは，化学物質のホルモン受容体を介した影響による極微量作動性の有無への疑問が発端であった．それはやがて「反応閾値の有無」への疑問へとつながり，それらの「相乗性・相加性の有無」，あるいは「高用量から低用量への外挿的推定の妥当性」や「反応の線形／非線形用量相関問題」などの諸問題に連関していった．やがて低用量問題として取り上げられた諸点が，相互に密接な関連をもったいわば“ひとつの問題”であることがわかってきた．だから解明の戦略は，その一角を取り崩すことであった．

2002年，ロンドン大学のコーテンカンプ(A. Kortenkamp)らは，内分泌攪乱性を有すると考えられる微量の物質のいくつかを一括して作用させたところ，個別に作用させたときには何らの影

[*3] 内分泌攪乱化学物質の疑いがあって作用機序の明らかでなかったものとしては，PCBsやPBBs，あるいは殺虫剤のうち塩素基や臭素基に置き換えたハロゲングループをもつような化学物質については，それらのフェノールの官能基の一部がステロイドホルモン受容体アゴニスト(作用物質)もしくはアンタゴニスト(競合物質)として働く性質があることがわかってきた．

[*4] 2000年10月，米国環境保護庁(EPA)は，いわゆる内分泌攪乱問題で対象となっているような物質影響が，通常の試験法で従来求められてきた無作用量(NOEL)や無毒性量よりも低い用量域で観察され得るかに焦点をあて，「低用量問題に関するワークショップ」をノースカロライナで開催した．そこでは，ビスフェノールA(BPA)の低用量データ報告の認否について，確認されたとする報告と認められなかったとする報告の双方に信頼性(credibility)を確認する結果となった．さらに低用量作用を示す試験の再現性や，長期試験がジエチルスチルベストロール(DES)にもBPAにも作用を示さなかった事実に言及し，低用量問題の不確実性を結論した．(http://www.epa.gov/scipoly/oscpendo/pubs/edmvs/lowdosepeerfinalrpt.pdf)

響を見せなかった用量の物質が，混合によって女性ホルモン(エストロゲン)にも匹敵する高いホルモン様活性を起こし得ることを試験管内反応で明らかにした[*5][(2)(3)]．個々ではわずかな影響に留まったとしても，複合した場合にこのような加算効果があるとすると，その影響はもはや無視できなくなると考えられる．これを相加性複合効果と呼んでいるが，かれらの実験のもうひとつのポイントは，限りなく閾値に近い低用量でこの相加性が認められたということである．続いて，個体レベルの試験系でも，ふたつのグループから顕著な相加効果が報告された[(4)(5)]．すなわちデンマークにおける2007年のワークショップ[*6]での発表で，クリスチャンセン(S. Christiansen)らは，抗アンドロゲン作用をもつビンクロゾリン，フルタミド，およびプロシミドンなどの農薬の複合投与で，尿道下裂や肛門生殖突起間長の短縮などが，混合体の投与動物群のみに見られたこと[(6)]を，ライダー(C. V. Rider)らは，そのビンクロゾリン，プロシミドンの他，リヌロンなどの農薬と，BBP, DBP, DEHPなどのフタレート類，あわせて7種の混合投与[*7]で，同様の指標による複合効果が観察された[(7)(8)]，と報告した．閾値付近での相加性の観察そのものが，従前では想定外な試験である．また，この結果は，フタレート類という類似の生体作用を有する物質の組合せゆえに認められた特異な現象とする考え方もあろうが，少なくとも類似のホルモン様作用シグナルに関する限り，複合効果を否定できないことが確定した点では，この結果のもつ意味は重く，今後のリスクアセスメント上，大きな検討課題を負うこととなった[(9)(10)(11)]．

種々のホルモンとホルモン類似物質による障害

以上のような認識に立って，身の回りの物質に目を向けてみると，われわれは，内分泌撹乱性が危惧される化学物質もさることながら，多くのホルモン物質，ホルモン類似物質そのものに取り囲まれて暮らしていることに気づく．もとより，生体ホルモンは，本来，低用量で作用し，また，用量，投与方法によっては有害になり得る性質のものである．したがって生体にはそのような影響を避けるためのより緻密な防護システムが備わっており，これが順調に機能していることが，外界から過度な影響を受けないようにする要件と考えられる．胎児の血清中に含まれる高濃度のα-フェトプロテインは，母胎間での女性ホルモンの影響を吸収する役割をもっているし，更年期女性に対するホルモン補充療法が，乳がんへのリスクなど様々の副作用を念頭において，ガイドラインに沿って慎重に行われることもそうした事情にもとづいている[(12)]．

これに対して，胚細胞期や胎児期・新生児期のように，まだ機能発達が安定する前の時点では，ホルモン様物質が生体ホルモンに置き換わって不可逆的な影響を及ぼすことが無視できない，とするデータが集積してきた[(13)]．この点は，思春期も同様と考えられる[*8]．こうした置き換え効果による障害や不全の可能性には，もとより注意が求められていた．ちなみに，ヒトでの発がん性に予防効果が期待され，ほぼ無制限に健康に良いかのごとくに理解されてきたいわゆる植物ホルモン(phytoestrogens)のひとつ，大豆イソフラボン[*9]

[*5] これはかつてタフト大学のソト(A. Soto)が試験管内の複合アッセイ系確立の可能性を論じた報告にならって，個体レベルでの影響を見たものである(A. M. Soto et al.: Environ. Health Perspect., **105**(3), 647(1997))．実際のデータは，著者らの文中にあるような相乗性(synergy)は意味せず，相加性(additive)に相当する．

[*6] 第4回内分泌撹乱物質ワークショップ(2007年5月28～31日)．デンマーク環境省の後援で，コペンハーゲンにて開催された．

[*7] フタル酸エステル類は，フタル酸とアルコールのエステル体で，ポリ塩化ビニルを主成分としたプラスチックの可塑剤として汎用されている．発生期の動物への曝露で，毒性，とくに生殖発生毒性が認められるため，フタル酸ビス(2-エチルヘキシル)(EDHP)をはじめとするフタル酸エステル類の玩具への使用は禁止されている．フタル酸エステル類の内分泌撹乱性は，女性ホルモン受容体への親和性が弱く確定していないが，何らかの複合的な影響の可能性を疑う研究者もあり，ここに示されるような複合影響の検討が行われてきた．最近，シャープ(R. M. Sharpe)らは周産期にフタル酸を曝露したラットにおける性分化の変調を報告し，注目されている(文献(8))．

[*8] EPAは思春期アッセイ試験の採用を重視しており，またこの点は，WHOのグローバルアセスメントでも，巻頭の要旨で取り上げられるべきであったと考えられる．

[*9] その功罪とも，糖質のはずれた大豆イソフラボン・アグ

の場合も，それまでの想定に反してその取り過ぎには，障害が惹き起こされる可能性が喚起されるようになっている*10．また，牛乳由来の調製乳を与えられた乳幼児と，豆乳由来の調製乳を与えられた乳幼児とでは，尿中のゲニスタインやダイゼインなどの植物ホルモン濃度は，後者は前者の500倍高いという報告[14]をあげて米国内分泌学会では注意を促している．こうした認識は，内分泌撹乱化学物質への理解の中で深まったもので，ホルモン様作用をもつ化学物質への注視は，もはやリスクアセスメントの必須要件となりつつある．

あらたに見いだされる低用量での生体影響

内分泌撹乱化学物質が，線形の用量反応関係をとらず，U字型や逆U字型の反応曲線をとったり，非常に低用量域で特異的反応を示すことについて，当初，そのこと自体に疑念を投げかける声も少なくなかった[15][16]．それはひとつに，当時その原理的説明がなし得ずいわば現象論に留まったこと，そしてなによりも安全性試験の領域で，非線形反応の想定される例を取り上げなかったからである．しかし様々の核内受容体やDNAの転写因子群の交差反応ネットワークを形成する受容体群や共役して働く補助因子での，至適の用量作動域がしばしば相互にずれていたりすることや，用量の増加とともに受容体反応が飽和に達し不応答状態になるといった現象が明らかにされるにつれて，問題点が整理され，急速に理解が進んでいる[17]．こうして理解のギャップの埋められた事柄は少なくないが，他方，まだ未知の領域にとどまるといわざるを得ない事柄も多い．米国の国立環境影響研究所(NIEERL)ではそうした点を重視して，これまでの研究計画のタイムラインを，より長期的展望をもって設定し，今後の検討に入っている[18]．

(1) 低用量域で観察される確率論的な生体反応

低用量影響の中には，種々の試験法を適用すると，しばしば意味のあるデータとは認識されず，いわばノイズのような結果として見られることが稀ではない．低用量での変化は頻度も低く，平均値をとるとしばしば測定のバラツキの中に隠れてしまうからである．内分泌撹乱現象が，特異な高感受性の遺伝的体質によって特定の個体に起こるものと考えて，たとえばエストロゲン受容体遺伝子多型を探索する研究も進められた．すると確かに先駆的な研究の中には，そうした原因形質が見いだされてきた[19]．しかし他方，非遺伝的に，確率論的に惹き起こされる可能性も見いだされている．この面からの研究は充分には進展していないが，ミシガン州立大学のグッドマン(J. Goodman)は，発がんプロモータの研究の中でつぎのような観察をしている．それは，フェノバルビタールによるDNAのメチル化という修飾の形成確率は，平均すれば実験群は対照群と差異が認められなかったが，ネズミ1匹ごとに検出してみると，対照群とは異なって，個体ごとに大きく変動した値が観察されたというものである[20]．DNAのメチル化は，エピジェネティックな変化と呼ばれるが，ここで認められた低用量におけるメチル化は確率論的に形成され，純系動物でも個体ごとに異なり同じ結果にはならない．このフェノバルビタールによるDNAのメチル化には，発がんのプロモータ作用が知られており，結果として，個体ごとに発がんの臓器分布や頻度が異なってくるという症状のランダムさを惹き起こすことになる．エピジェネティックな変化としては，他に，クロマチン凝縮，ヒストン修飾などがあるが，内分泌撹乱化学物質における低用量反応に対しても，こうしたエピジェネティックな現象として理解する考え方が急速に進展している[21]．これこそ従来の毒性学が想定してこなかった現象で，この領域での今後の進展に注視する必要がある．なお，こうしたエピジェネティックな変化がゲノムに刷り込まれて(＝ゲノムインプリンティング)，世代を超えて伝達され，固定されてゆく可能性も現実の問

リコンのエストロゲン類似作用に関連するものと考えられている．

*10 厚生労働省：大豆及び大豆イソフラボンに関するQ&Aを参照．(http://www.mhlw.go.jp/houdou/2006/02/h0202-1a.html)

題となっている[22][23].

(2) 高感受性期: 胎生期・新生児期・思春期の問題

機能的に安定する前の胎生期での影響に関して，無視できない不可逆的な事象が指摘されていることは前述した[24]．胎生期・新生児期・思春期問題には，低用量問題との関連を示すデータが少なからず見いだされており，WHOの報告書「グローバルアセスメント」[25]でも指摘された通り，胎児や新生児では，ウィンドウ効果*11と呼ばれるわずかな期間での投与が特異的な不可逆反応を惹き起こす現象が知られている[26][27]．また，野生型の成体では検知されない用量レベルだが，遺伝子改変動物などを用いた過剰反応系の動物を用いると検出される，“新しい概念の影響”の観点から，①閾値問題，②非線形の用量相関，あるいは③相加反応などの問題を見直す試みも進んでいる．内分泌攪乱化学物質として危惧される物質の生体影響研究では，影響メカニズムが未解明である一方，確認や追試が必要となることも少なくない．とくに時を経て遅れて現れる成長後の行動にかかる影響については，解析法そのものに未知の点が少なくない．系統的な実験的情報収集が求められる所以である．

WHOの「グローバルアセスメント」では触れられなかったが，性ホルモンのバランスの不安定な“思春期”に関する研究も，胎生期・新生児期と同様に注意が払われるべきと考えられる．胎生期や思春期などの性成熟の臨界期への曝露が与える影響の評価基準は，いまだ定まっていない．疫学的に尿道下裂の発症に関与する遺伝子*CXorf6*がクローニングされ[28][29]，実験的には胎生期へのビスフェノールAの投与が思春期の早発傾向とつながるとの報告もなされている[30]．*CXorf6*のような遺伝子と化学物質との持続的な相互作用など，今後の研究が求められている．

*11 形態形成期である胎生期の狭い胎齢期間に，異物投与期特異的に生体影響が観察されること．

(3) 内分泌器官の拡張や，内分泌機能の概念の拡張

この10年余りの研究により，従来，性ホルモン受容体では想定されていなかった細胞内器官や組織に，内分泌器官の役割が，見いだされてきた．トマス(P. Thomas)らによる膜受容体*12の同定は，そのカテゴリーに含まれる発見であった[31][32]．この発見は，オルファニデス(G. Orphanides)らによって指摘されていた，従来の性ホルモン受容体機能に一般的であった核内受容体で説明の困難だった即時型反応を，遺伝子発現を介さないノンゲノミック(non-genomic)な機構にもとづくホルモン様作用[33]で理解するうえで決定的な役割を果した．やがて，細胞小器官である小胞体の膜にもエストロゲン受容体(ER)の局在が見いだされ[34]，急峻な反応への対応機構が明らかになっていった．これらの発見は，内分泌攪乱問題には，多くの未知の要因が関与していることをあらためて喚起した．肝臓や，脂肪細胞など，これまで内分泌器官とは考えられてこなかった臓器が，内分泌器官としての役割を果たしていることも明らかになってきた．たとえばノニルフェノールという物質は，通常の方法で見るとごく弱い女性ホルモン様の作用をもっているにとどまるが，肝臓に注目すると，生殖器よりもずっと強い活性を示すことが，岡崎国立基礎生物学研究機構の井口泰泉らによって明らかにされている[35]．これは，“内分泌器官としての肝臓”という見方につながる結果である．脂肪組織についても同様のことが指摘されている[36]．イボニシでの内分泌攪乱の知られる有機スズが脂肪組織の増殖を惹き起こすことや，それらの機構に核内受容体が関与していることなどは，これに関連するかもしれない．かくして，同じ受容体結合能を有するリガンド物質が，広範な標的受容体シグナル機構，さらにはまったく異なった表現型(フェノタイプ)の発現に関わる共働

*12 核内受容体と区別する．核内受容体が細胞内で，細胞質や核内にあって，特異的リガンド物質をDNAと特異的に相互作用を促し，転写に寄与するのに対して，細胞膜上に分布し，核酸と直接的相互作用を介さずにホルモン受容体影響を惹起する．

補助因子などとの相互作用を惹き起こすことなど，つい先頃までの認識を書き換える驚くべき関係が浮かび上がっている．

内分泌機能をもつ新しい器官の発見に加えて，内分泌器官そのものの概念を変える事象も見いだされている．異物受容体と呼ばれているダイオキシン受容体は，エストラジオールが存在しない状態では，P300と名付けられているタンパク分子の助けで転写活性化を担って，女性ホルモン様の作用をもつことが東京大学分子細胞生物学研究所の加藤茂明のグループによって発見された[37]．しかもここでは，エストラジオールがあるときは，この分子は，反対にユビキチン・リガーゼ(Ubiquitin ligase)と呼ばれる複合体を形成し，エストロゲン受容体を壊して，抗女性ホルモン様の役割を発揮する[38]．これは，ホルモン受容体でもない，異物受容体と呼ばれる生体内分子が，種々のホルモン様の作用を時に機能を変化させつつ発揮するということである．内分泌撹乱問題の分子的基盤が，概念的に大きく拡大しているものと考える所以である．内分泌系の拡がりを認識すれば，この発見の示唆する重みに改めて驚かされるであろう．

「生体調節障害の毒性学」を確立するために

内分泌撹乱化学物質問題が取り上げられるきっかけになったことそのものは，ヒトや野生生物の生殖や内分泌機能に関する危惧にあった．やがてその可能性の原点がホルモン作動性の化学物質の低用量での影響にあるものとの認識に近づいた．しかしこれは従来の試験法では有害性が観察されないなど，その背景となるメカニズムがなかなか明らかにならなかった．

すでに見たように，従来の試験法で観察されなかった背景は，この低用量作用が，従来の試験法が対象としていた毒性とは異なった生体障害機構にもとづくものであったためだった．内分泌撹乱化学物質では，生体内分子の壊変や変質などといった構造異常の前に，むしろ曝露影響は，低用量であるがゆえに通常の生体の生理的調節水準下で目に見えない形で微視的機能不全へと進行し，エピジェネティックに，次第に持続的調節不全に陥る生体異物相互作用の調節異常にもとづくのである．これまで注目されてこなかった事柄であり，事実，受容体を介した諸々の影響に焦点は収束しつつあったが，それらがどのように障害に結びつき得るかは，想定にとどまっていた．それは明らかに従来型の，生体分子の酸化や還元，DNAや脂質などの高分子への付加体形成や架橋形成などの化学的修飾，主として生体物質の壊変，変質といった化学反応を基礎とした直接的構造変化とは異なっており，それらに主眼をおいて作られてきた試験法では評価できなかったということであったと考えられる．

この問題の発端の頃，頻度の低い，胎生期のような形態形成期の事象や，小児の生殖器系に限局した内分泌撹乱現象を，稀な確率論的現象と解釈する報告がなされたことがあった．これも以上のような背景と結びついていたものと考えられる．裏返せば，フィードバック機構や“可逆性”の背後で，むしろ低用量曝露にあってはじめて惹き起こされるこの種の毒性は，それまでの毒性試験では検討されず，想定されていなかった．事実，先頃出版されたタイル(R. W. Tyl)らによるビスフェノールAの2世代試験でも，従来の毒性試験にない幅広の用量点をとった試験であったにもかかわらず，何らの影響も認められなかった[39]．タイルらの試験法と，この間工夫を重ねて行われた一連の研究における実験条件との違いには，前者における，持続投与によって惹き起こされるウィンドウ効果の棄却や，被験動物に不応性が導き出されることなどがあるものと推定されるが，この試験法の無力については，背景の解明，両者の相違の可視化のために，胎生期の狭い期間に限局した特異的な遺伝子発現と，投与異物との相互作用の詳細などが明らかにされる必要がある．

低用量における生体異物相互作用の調節障害は，内分泌撹乱化学物質問題を契機として見いだされた，これまでの毒性学の標的に含まれない生体障害性を基礎としたあたらしい概念の毒性現象であ

る．だから従来の毒性学の方法論に加えて，独自に進めるべき研究課題を含んでいる．たとえばこれらの調節障害を原理とした有害性では，通常の調節が生理的範囲から異常状態に移行する過程が，従来の表現型による線引きの困難ないわば振幅の変化のような，境界を含んでいる．そうした境界には直接的な構造異常が伴っていないようであり，これを裏付ける生体分子シグナル機構のより詳細な研究が必要である．こうした事柄は，あたらしい毒性学で求められるこれまでにない課題である．内分泌攪乱化学物質問題によってはじめて見いだされたこのあたらしい生体障害の概念は，当初の想定を超えて，一般論として生体調節障害の全域に及ぶ課題の拡がりを内包している．この点は，米国内分泌学会の内分泌攪乱物質に関する最近発表されたはじめての公式声明の冒頭でも指摘されている[40]．“化学物質の生体調節障害”という課題を対象としたあたらしい毒性学の確立のためには，さらなる概念の構築・整理と，対応する試験法の樹立を一層確かなものとする必要がある．

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Review

AhR acts as an E3 ubiquitin ligase to modulate steroid receptor functions

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ABSTRACT

The arylhydrocarbon receptor (AhR) mediates the adverse effects of dioxins, including modulation of sex steroid hormone signaling. The role of AhR as a transcription factor is well described. AhR regulates the expression of target genes such as CYP1A1; however, the mechanisms of AhR function through other target-selective systems remain elusive. Accumulating evidence suggests that AhR modulates the functions of other transcription factors. The ligand-activated AhR directly associates with estrogen or androgen receptors (ERα or AR) and modulates their function both positively and negatively. This may, in part explain the sex steroid hormone-related adverse effects of dioxins. AhR has recently been shown to promote the proteolysis of ERα/AR through assembling a ubiquitin ligase complex, CUL4B^{AhR}. In the CUL4B^{AhR} complex, AhR acts as a substrate-recognition subunit to recruit ERα/AR. This action defines a novel role for AhR as a ligand-dependent E3 ubiquitin ligase. We propose that target-specific regulation of protein destruction, as well as gene expression, is modulated by environmental toxins through the E3 ubiquitin ligase activity of AhR.

Contents

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Abbreviations: AhR, arylhydrocarbon receptor; ERα, estrogen receptor; AR, androgen receptor; XRE, xenobiotic-responsive element; ERE, estrogen-responsive element; bHLH/PAS, basic helix-loop-helix/Per–Arnt–Sim; AF-1, autonomous activation function; E_2, 17β-estradiol; 3MC, 3-methylcholanthrene; βNF, β-naphthoflavone; CRL, cullin–RING ubiquitin ligase; SCF, Skp1–CUL1–F-box; CUL4B, cullin 4B; DDB1, damaged-DNA-binding protein 1.

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1. Introduction

Dioxin-type environmental contaminants, such as tetrachloro-dibenzo-*p*-dioxin (TCDD), exert toxic effects [1]. Some of these toxicities are estrogen- and androgen-related actions [2–7]. The arylhydrocarbon receptor (AhR) is a ligand-dependent transcription factor belonging to the basic helix-loop-helix/Per–Arnt–Sim (bHLH/PAS) family. AhR possesses a variety of biological and toxicological functions [8–11] (Figs. 1 and 2). AhR mediates the toxicological effects of dioxins. In addition, AhR plays a physiological role in various tissues such as the reproductive and immune systems. The transcriptional activity of AhR is regulated by direct binding of its ligands [12,13] (Figs. 1 and 2A). The unliganded AhR is sequestered in the cytosol by interacting with the Hsp90/XAP2 (also called as ARA9 or AIP) chaperon complex [8–11]. Ligand binding to the PAS-B region of AhR is thought to induce conformational changes and subsequent translocation of the AhR complex to the nucleus [8–10]. AhR then dimerizes with the AhR nuclear translocator (Arnt) in the nucleus after dissociating from the chaperon complex, recognizes the xenobiotic-responsive element (XRE), and recruits co-activators such as the histone acetyltransferase p300/CBP, chromatin remodeling factor Brg1, and the mediator (DRIP/TRAP) complex to activate transcription [8–10] (Fig. 1). The AhR/Arnt heterodimer induces the expression of target genes, such as CYP1A1, CYP1A2, and glutathione-S-transferase [1].

The actions of the direct target genes of AhR alone do not fully explain its toxicological and physiological effects. Accumulating evidence suggests that the AhR exhibits its regulatory functions by modulating the function of other transcription factors [2,11], including estrogen receptor (ERα and ERβ) [14–19] and androgen receptor (AR) [18,19] (Fig. 1). These cross-talk pathways are important mediators of the functions of endogenous and exogenous AhR ligands. The liganded AhR recently has been shown to promote the ubiquitination and proteasomal degradation of ERs and AR by assembling a ubiquitin ligase complex, $CUL4B^{AhR}$ [18,19]. Thus, complexes of the AhR with ERs or AR appear to regulate transcription as functional units by multiple mechanisms. In this review, we will summarize a novel role for AhR as a component of an E3 ubiquitin ligase complex, which mediates cross-talk of AhR with sex steroid receptors through promotion of proteolysis.

2. Cross-talk of AhR with ERs or AR

2.1. *Transcriptional regulatory mechanism involving nuclear receptors*

ERs and AR belong to the nuclear receptor superfamily of transcription factors [20–22] (Fig. 2). Nuclear receptors, by acting as ligand-dependent transcription factors serve as

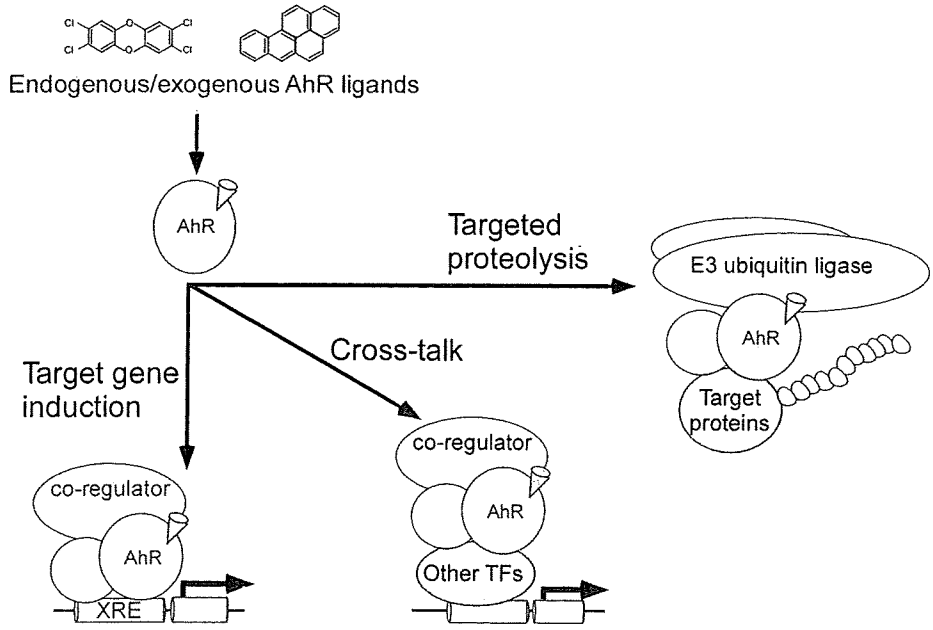

Fig. 1 – Different modes of the AhR signaling pathways. Molecular pathways for AhR-mediated biological actions. AhR may exhibit its biological actions through different modes of pathways as illustrated. Typically, AhR directly binds to its target gene promoters and induces expression of these genes. In addition, cross-talk of AhR with other transcription factors, as well as the function of AhR as an E3 ubiquitin ligase, is considered important for AhR biology. XRE, xenobiotic-response element; TF, transcription factor.

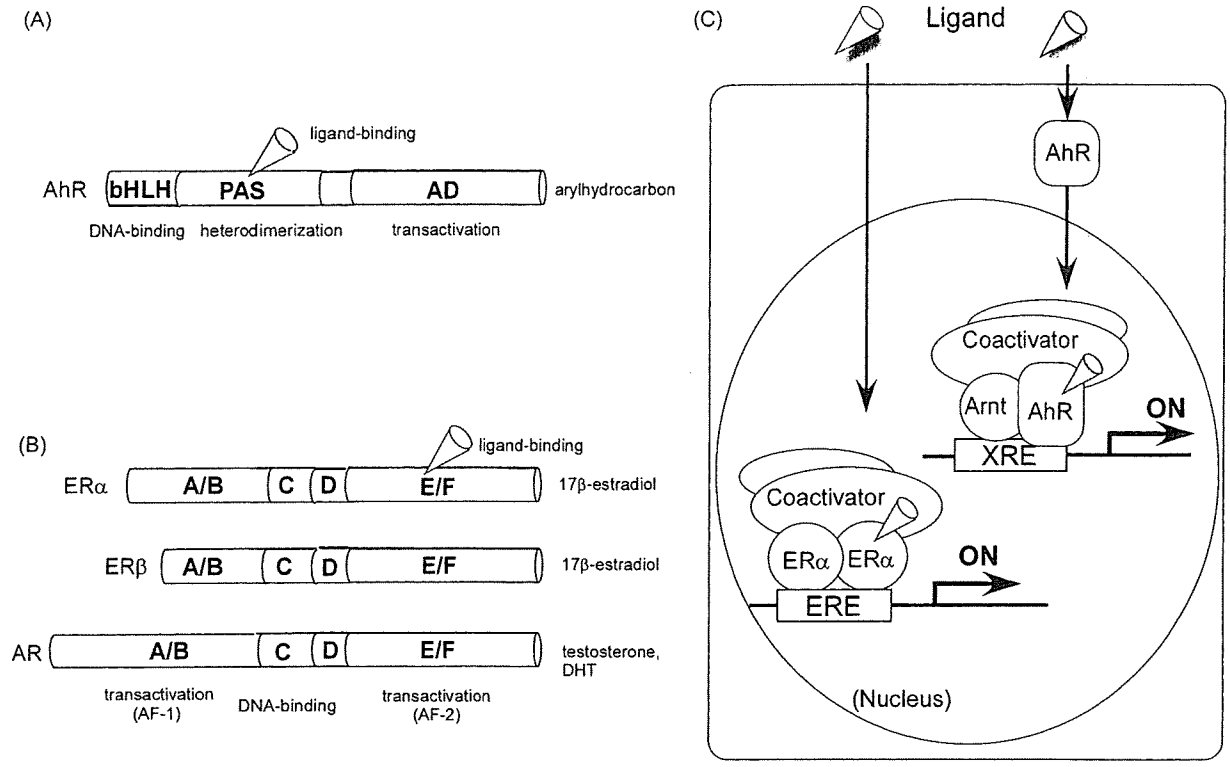

Fig. 2 – Structure and molecular mechanism of AhR and nuclear receptors. A and B domain structures of AhR (A) and nuclear receptors (B). Domain architectures and cognate ligands for these receptors are illustrated. bHLH, basic helix-loop-helix; PAS, Per-Arnt-Sim domain; AD, activation domain; AF, activation function; DHT, dihydrotestosterone. (C) Mechanisms of gene regulation mediated by AhR and nuclear receptors. ERE, estrogen-response element.

sensors for low molecular weight, fat-soluble ligands such as steroids/thyroid hormones, and vitamins A and D [20,21]. Members of the nuclear receptor gene superfamily share a common domain structure with distinct functional domains, designated A–E [21] (Fig. 2B). The ligand-binding domain is located in the C-terminal E domain. The most conserved C domain, located in the middle of the receptor, serves as the zinc finger-type DNA-binding domain. This domain specifically recognizes its cognate response elements in the target gene promoters. The N-terminal A/B domain and the C-terminal E domain are required for ligand-induced nuclear receptor transactivation functions. The autonomous activation function (AF-1) in the A/B domain is constitutively active but is presumably masked in the absence of ligand. The autonomous activation function (AF-2) in the ligand-binding E domain is, in contrast, dependent on ligand binding through the ligand-dependent conformational change of helix 12 and subsequent formation of a hydrophobic surface for the interaction with co-regulators [20] (Fig. 2).

Ligand-bound nuclear receptors recruit a number of transcriptional co-regulators and co-regulator complexes to the target gene promoters to mediate ligand-dependent transcriptional control [21,22] (Fig. 2). These complexes can be classified into three categories according to their functions. The first class of co-regulator complexes modifies histone tails covalently [23]. The amino-terminal tails of histones are subjected to various covalent modifications such as acetylation, methylation, phosphorylation, and ubiquitination by specific histone-modifying enzymes. These post-translational histone modifications are thought to serve as a 'histone code' that fine-tunes the transcriptional state through chromatin structure rearrangement [23]. The second class of complex mediates ATP-dependent dynamic remodeling of chromatin structure [22]. Chromatin remodeling complexes use ATP hydrolysis to rearrange nucleosomal arrays in a non-covalent manner. These chromatin remodeling complexes support the accessibility of co-regulator complexes and transcription factors to specific promoter regions. The last co-regulator complex class, the mediator complex, directly regulates transcriptional control by physically interacting with general transcription factors and RNA polymerase II. Recent evidence suggests that numerous co-regulators and nuclear receptors are recruited onto the promoters in an ordered manner, associating and dissociating transiently [24,25]. Nuclear receptors, as well as other transcription factors, serve as specific adaptors that connect co-regulator complexes and specific promoter regions.

The ligand-dependent nuclear receptor function is also regulated by other classes of signal transduction pathways. Such cross-talk pathways include at least two mechanisms: functional modulation through post-translational modification, and the association with other classes of transcription factors. MAPK, activated by EGF, phosphorylates ERα at serine 118 [26]. This in turn potentiates the ligand-dependent transactivation function of ERα [26] as well as its rapid turn-over. Phosphorylation-mediated functional modulation has been reported for a number of nuclear receptors to date.

Complex formation-based cross-talk mechanisms are also seen in several nuclear receptors including the glucocorticoid receptor (GR) [27]. GR ligands have an anti-inflammatory action, which is mediated through ligand-dependent repression of AP-1 activity through direct association. More recently, the exchange of different classes of co-regulator complexes has been reported to underlie the signal cross-talk pathway. Ligand-activated PPARγ typically assembles co-activator complexes on its cognate promoters. In the repression of NF-κB activity, PPARγ forms a complex with NF-κB, and this complex stably associates with an NCoR co-repressor complex by inhibiting the degradation of NcoR [28]. A current view of signal cross-talk at the transcription levels is that signal/ligand-dependent transcription factors associate with each other to assemble diverse types of co-regulator complexes. These exchange dynamically and regulate transcription in a manner specific for each cross-talk pathway [22].

2.2. *Molecular mechanisms of cross-talk of AhR with estrogen or androgen receptors*

Signal cross-talk pathways are important mediators of the functions of AhR ligands in various tissues. Dioxin-type environmental contaminants exert both estrogen- and androgen-related effects [1–3,5–7,29–32] (Fig. 3). Dioxins have well-described anti-estrogenic effects, such as the inhibition of estrogen-induced uterine enlargement, MCF-7 cell growth, and target gene induction [3,7]. However, there is also evidence to the contrary as dioxins have also been shown to have estrogenic effects including the stimulation of uterine enlargement [29], induction of estrogen-responsive genes such as *VEGF*, *c-fos*, and *TERT*, and a similar pattern to estrogen of transcriptional regulation in a genome-wide study [6]. In addition, AhR-deficient mice exhibit impaired ovarian follicle maturation [33]. Using AhR-deficient cells, the importance of AhR in the proliferation of mammary cells has been confirmed [34]. These findings suggest that AhR, activated by its endogenous ligand, may modulate the estrogen signaling pathway. Similarly, dioxins exert both androgenic and anti-androgenic effects on prostate development in an age-specific manner [5]. As is true for other cross-talk pathways [22], the AhR appears to modulate estrogen/androgen signaling both positively and negatively depending on cellular context.

The molecular mechanisms of AhR modulation of ERα have been extensively studied, and both direct and indirect regulatory mechanisms have been proposed. First, TCDD/AhR either increases or decreases estrogen levels through an indirect mechanism [2,35]. TCDD promotes the clearance of estrogen, thereby repressing ER transcriptional activity [35]. AhR-deficient mice have decreased estrogen production due to impaired induction of aromatase (CYP19) gene expression [33]. Another indirect mechanism involves competitive DNA binding of AhR and ER on the responsive promoters [2]. AhR and ER, each bound to its own target promoter recruits transcriptional co-regulators

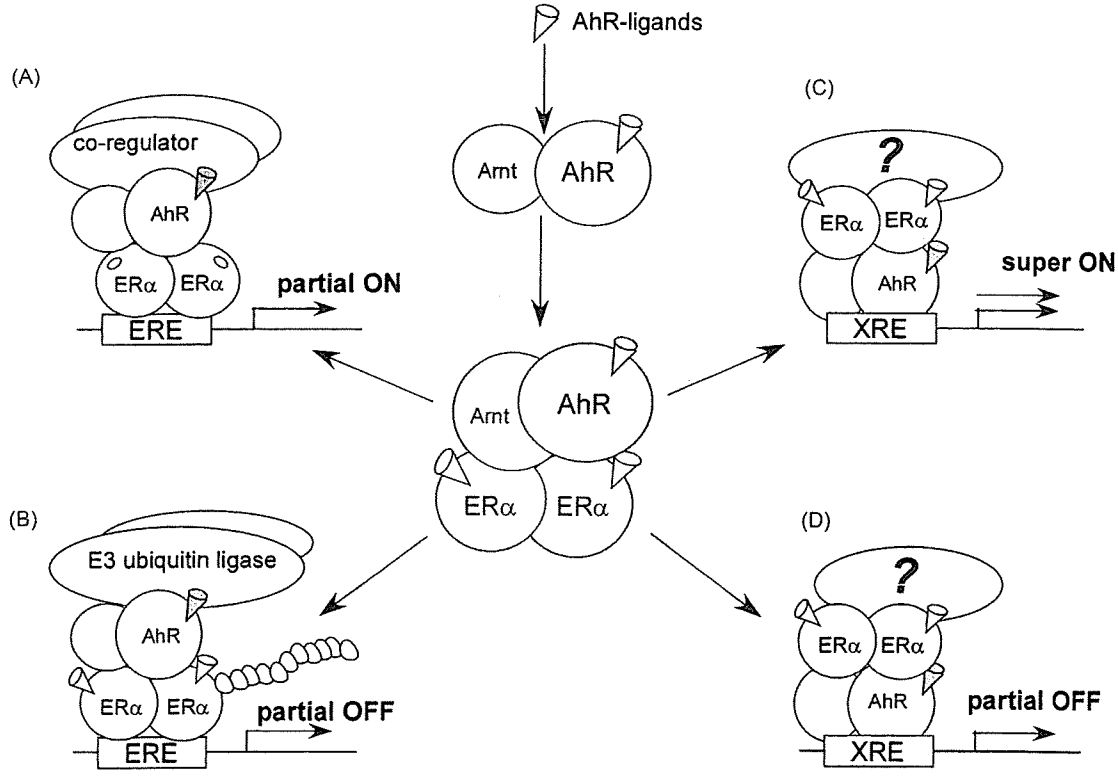

Fig. 3 – Cross-talk of AhR with ERα through direct association. Ligand-bound AhR directly associates with estrogen or androgen receptors (ERα, ERβ, or AR) in the nucleus. This association leads to different types of cross-talk between AhR and ERs/AR, as illustrated (see text for details). (A) Ligand-bound AhR associates with unliganded ERs upon ERE and recruits transcriptional co-activators. (B) Ligand-bound AhR forms E3 ubiquitin ligase complex and recognizes ERs for proteolysis. (C) Ligand-bound ERα associates with AhR and activates transcription through XRE. (D) Association of ERα with AhR results in repression through XRE.

in a competitive manner. This mechanism may be limited to specific genes and conditions since not all of the estrogen-responsive promoters contain XRE.

More recently, direct association of AhR with ERs has been independently reported. Ligand-activated AhR/Arnt associates with ERα and ERβ through the N-terminal A/B region within ERs [14–18] (Fig. 3). By means of this association, the liganded AhR potentiates the transactivation function of 17β-estradiol (E_2)-unbound ERα (Fig. 3A), while it represses E_2-bound ERα-mediated transcription upon the estrogen-responsive element (ERE) [14] (Fig. 3B). The interaction of AhR/ER is induced by different AhR ligands, such as TCDD, 3-methylcholanthrene (3MC), and β-naphthoflavone (βNF). The activation of AhR is thought to be sufficient for the interaction with ERα, as a constitutively active form of AhR [12] modulates ERα function in the absence of AhR ligand [19]. These results suggest that the cross-talk of AhR with ER is initiated primarily through stimulation of AhR. Supporting this, ERα is predominantly located in the nucleus, whereas AhR translocates to the nucleus upon ligand stimulation. The association of AhR/ERα has been shown by several independent approaches, including *in vitro* [36], *in vivo*, and biochemical methods [18]. Moreover, AhR/ERα cross-talk in the transcriptional regulation of ERα-responsive genes is abolished in AhR-deficient mice [10,33], confirming the specificity of the molecular pathway *in vivo* [14]. Reciprocally, E_2-bound ERα associates with XRE-bound AhR to either potentiate [15] (Fig. 3C) or repress [16] (Fig. 3D) AhR-mediated transcription. Considered together, the AhR/ERα complex may be able to bind to either XRE or ERE through the attachment functions of AhR or ERα, respectively. Alternatively, different complex subtypes that contain AhR/ERα may control promoter selectivity (Fig. 3). Reflecting this functional cross-talk, Arnt also acts as a co-regulator for both ERα and ERβ [37].

The proposed mechanism of AhR/ER association is a reasonable explanation for dioxin/estrogen cross-talk. First, this mechanism explains the functional AhR/ER cross-talk irrespective of differences in target gene promoters. Second, ligand-dependent AhR/ER association may result in a rapid cellular response to dioxins in terms of ER activity. The responses of ER transcriptional activity to AhR ligands are observed within a few hours in cultured cells as well as in mice, which supports the existence of direct cross-talk mechanisms. Third, variations in the AhR/ER containing co-regulator complexes may result in the complex, bi-phasic consequences of AhR/ER cross-talk. Given that complexes containing different classes of transcription factors can recruit co-regulator complexes distinct from their cognate associating complexes [22], it is possible that the AhR/ER complex, acting as a functional unit, may recruit different types of complexes depending on the cellular context. A current area of interest is the identification of the molecular determinants by which the activity of the AhR/ER complex is controlled.

3. Ubiquitin ligase activity of AhR

3.1. *The ubiquitin–proteasome system*

The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels in response to various cellular contexts in metazoans (Fig. 4A). Whereas the transcriptional regulatory system is targeted by environmental fat-soluble ligands, the involvement of the ubiquitin–proteasome system in the adverse effects of these environmental toxins remains largely unknown. The target selectivity of these systems depends on the recognition of specific DNA elements by sequence-specific transcription factors [20–22] and recognition of degradation substrates by E3 ubiquitin ligases [38–41] (Fig. 4B). These transcription factors and E3 ubiquitin ligases primarily serve as specific adapters to subsequently recruit enzymes such as transcriptional co-

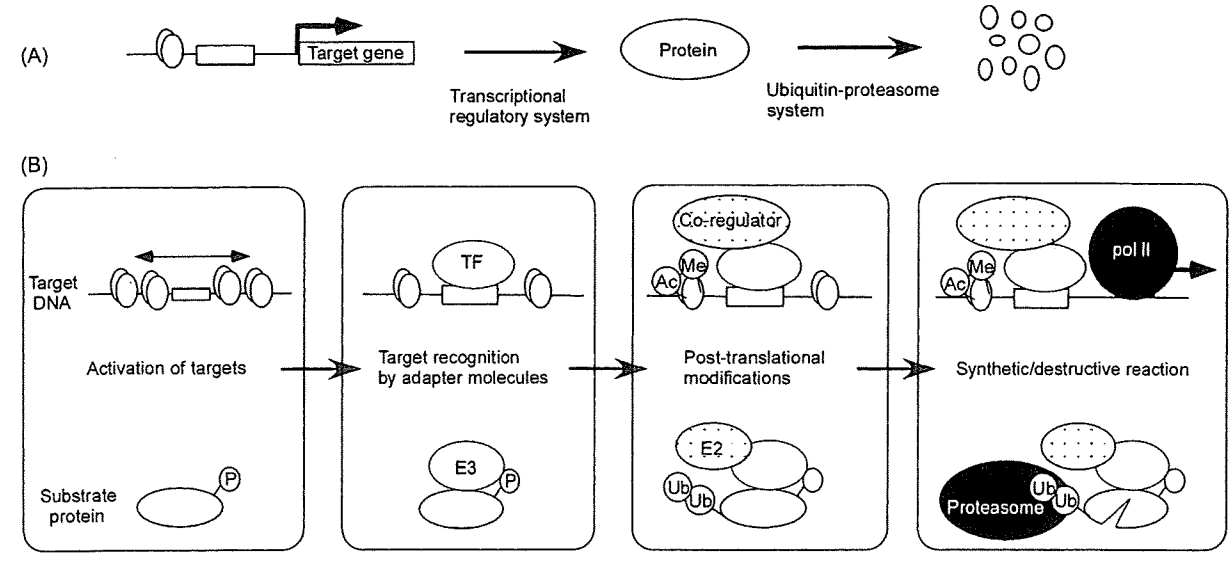

Fig. 4 – The ubiquitin–proteasome system. (A) The transcriptional regulatory system and the ubiquitin–proteasome system are two major target-selective systems that control intracellular protein levels. (B) The transcription factors and E3 ubiquitin ligases primarily serve as target-specifying adapters in these systems. Ub, ubiquitin; P, phosphorylated serine/threonine; Ac, acetylated lysine; Me, methylated lysine; Pol-II, RNA polymerase II.

regulators and E2 ubiquitin-conjugating enzymes, respectively, to appropriate targets. Considering the functional analogy of E3 ubiquitin ligase and transcription factors, it is possible that E3 ubiquitin ligase also serves as a target of environmental toxins.

The ubiquitin–proteasome system, which regulates cellular protein degradation, plays a pivotal role in cellular homeostasis [38–41]. Ubiquitin is a 76 amino acid polypeptide that is highly conserved among eukaryotes. Ubiquitin is covalently attached to lysine (Lys) residues of substrate proteins. Ubiquitination of proteins is catalyzed by sequential reactions involving ubiquitin activating enzyme (E1), ubiquitin-conjugating enzyme (E2), and ubiquitin protein ligase (E3). Ubiquitin is conjugated either as one molecule (mono-ubiquitination) or as a tandem polymer (poly-ubiquitination). Poly-ubiquitination can occur at any of seven lysine residues in the ubiquitin molecule. The Lys48-linked poly-ubiquitin chain is then recognized by the 26S proteasome for subsequent proteolysis (Fig. 4B).

Among E1, E2, and E3 enzymes, the E3 ubiquitin ligases are most diverse and therefore possess substrate specificity. E3 acts as a bridge between E2 and the substrate, maintaining the appropriate distance. E2 then conjugates ubiquitin to the substrate [38–41]. Of the RING-type E3s, the largest class is comprised of the cullin–RING ubiquitin ligases (CRLs) [40–44]. CRLs are multisubunit complexes that include a cullin (CUL1, 2, 3, 4A, 4B, or 5) subunit, a RING finger protein Rbx1/Roc1 or Rbx2/Roc2, and a substrate-recognition subunit. Cullin serves as a scaffold protein, binding to the substrate-recognition subunit or adapter protein at its N-terminus while binding to Rbx1 at its C-terminus [41]. Rbx1 binds to E2 enzymes through RING finger to support efficient conjugation of ubiquitin to the substrates. Their diverse substrate-recognition subunits enable CRLs to target numerous substrates. The best characterized CRLs are the SCF (Skp1–CUL1–F-box) complexes. In SCF complexes, F-box proteins function as a substrate-recognition subunit by binding to Skp1, which is bound to the N-terminal region of CUL1. F-box proteins and other types of substrate-recognition subunits serve as adapters for target-specific substrates. Therefore, any protein binding to E3 core components can potentially act in a manner similar to substrate-recognition subunits. More interestingly, F-box proteins and other types of substrate-recognition subunits are rapidly degraded through an auto-catalytic mechanism once they are integrated into the CRL core complexes [42]. In this way, CRLs can efficiently ubiquitinate different substrates by associating with different substrate-recognition subunits. This raises the possibility that F-box and F-box 'equivalent' proteins act either as substrates or as adapter components, as in the case of DDB2 in the CUL4-based CRL complex [45–50].

3.2. *AhR is an E3 ubiquitin ligase*

As discussed above, dioxins, through activating the AhR, have well-described effects on the transcriptional regulatory system. TCDD is also reported to decrease the uterine ERα protein level in the rat [51], suggesting that AhR may also be involved in the control of protein stability. Somewhat unexpectedly, our own study has shown that in a ChIP analysis, the ligand-bound AhR does not block co-activator recruitment of liganded ERα. In addition, repression of ERα transcriptional activity by AhR is not observed when ERα is over-expressed in transient reporter assays (Ohtake et al., unpublished data). These observations imply that the ligand-activated AhR has an additional molecular role beyond transcriptional regulation, at least in the modulation of sex hormone signaling.

Exploring the functions of AhR in sex hormone signaling, we found that upon activation of AhR by binding of AhR ligands such as 3MC and βNF, as well as by expression of constitutively active AhR, protein levels of endogenous ERα, ERβ, and AR, were drastically decreased without alteration in mRNA levels [19] (Fig. 5). Since ligand-bound AhR and ERα proteins are ubiquitinated for proteasome-mediated degradation [52–57], we tested whether the functional modulation of ERs and AR by activated AhR is related to this degradation system. 3MC-enhanced degradation of sex steroid receptors is attenuated in the presence of a proteasome inhibitor MG132, and 3MC-enhanced poly-ubiquitination of ERα is consistently observed irrespective of E2 binding. MG132 treatment abrogates the transcriptional modulation of liganded sex steroid receptor function by activated AhR. This indicates that the ubiquitin–proteasome system mediates the repressive AhR–ER cross-talk pathway.

These experiments provide evidence that AhR acts as an E3 ubiquitin ligase component. First, FLAG–AhR immunoprecipitated complexes exert a self-ubiquitination activity in an E1/E2 enzyme-dependent manner *in vitro*. Second, 3MC-dependent recognition of ER and AR by AhR [14] appears to induce ubiquitination of ER/AR. Third, degradation of AhR itself is accelerated upon activation of degradation of sex steroid receptors, which is a typical sign of self-ubiquitination of the E3 component [42]. Taken together, these properties of AhR resemble that of classical adapter components of the E3 ubiquitin ligase complex such as F-box proteins in the SCF complex [39,42], DDB2/CSA in the CUL4A complex [45–49], and VHL in the CUL2 complex [58]. Therefore, we reasoned that activated AhR might serve as an E3 ubiquitin ligase component.

Supporting this idea, an AhR associating ubiquitin ligase complex has been biochemically purified [59] from HeLa cells. This complex includes cullin 4B (CUL4B) [39,60], damaged-DNA-binding protein 1 (DDB1) [61,62], and Rbx1 [39] together with subunits of the 19S regulatory particle (19S RP) of 26S proteasome as well as Arnt and transducin-beta-like 3 (TBL3) (Fig. 5). The core complex appears to constitute a CRL-type E3 ligase, and therefore is referred to as $CUL4B^{AhR}$. Although the typical CUL4B-type CRL complex contains substrate-recognition components having a WDXR/DWD motif [45–49], no such component has been identified in this complex. AhR directly interacts with the N-terminal region of CUL4B in GST pull-down assays. Together with the direct interaction of AhR with ER, it appears that AhR may act as a substrate-recognition component in the $CUL4B^{AhR}$ complex. Using an *in vitro* reconstituted ubiquitination assay, the E3 ubiquitin ligase activity of $CUL4B^{AhR}$ for ERα is dependent only on 3MC, and not on E_2. This suggests that $CUL4B^{AhR}$ has the unique property of being able to respond to ligand signals by complex assembly and ubiquitin ligase activity (Fig. 5). The importance of the $CUL4B^{AhR}$ components for the promotion of ERα ubiquitina-

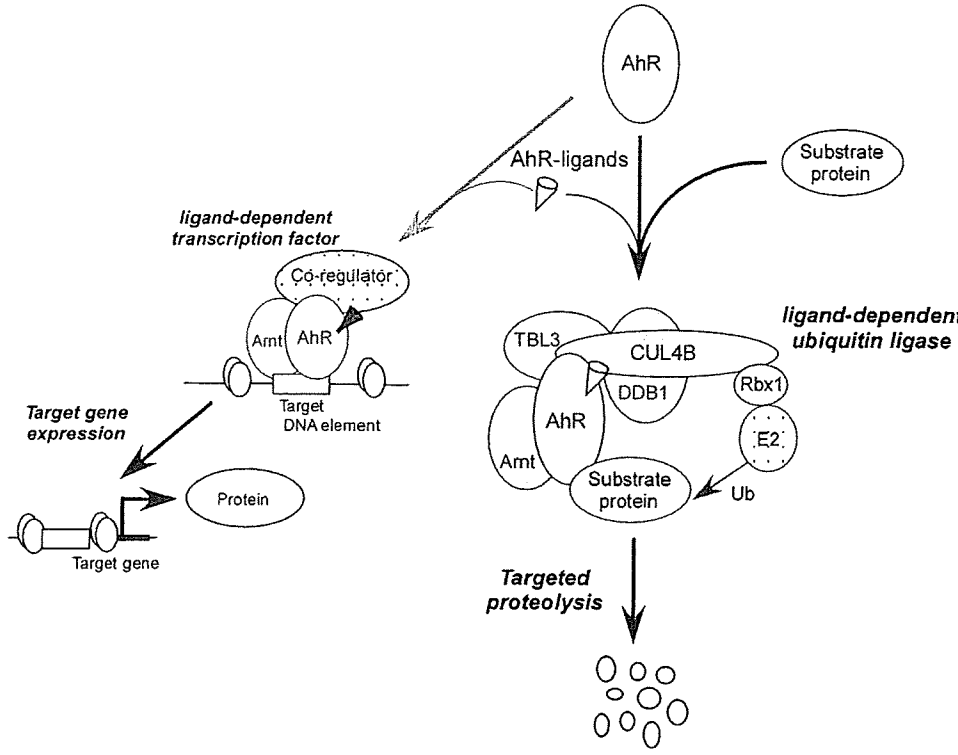

Fig. 5 – An E3 ubiquitin ligase activity of AhR. Ligand-bound AhR assembles a CUL4B-based atypical E3 ubiquitin ligase complex, $CUL4B^{AhR}$, to mediate a non-genomic signaling pathway of fat-soluble ligands. AhR serves as a ligand-dependent ubiquitin ligase, as well as a transcription factor (see text for details). DDB1, damaged-DNA-binding protein 1; TBL3, transducin-beta-like 3.

tion and degradation has been demonstrated in knock-down experiments. Degradation of ERα or AR in the uterus and prostate is inducible by treatment with AhR ligands. Such degradation of ERα or AR is not seen in AhR-deficient mice [10,33]. This confirms that the AhR has E3 ubiquitin ligase activity *in vivo*. The anti-estrogenic effects of AhR ligands on estrogen-dependent uterine cell proliferation [14] appear to be mediated by the E3 ubiquitin ligase activity of AhR.

3.3. *Perspectives on the E3 ubiquitin ligase activity of AhR in cross-talk pathways*

Although it is well established that AhR is a key factor in mediating the adverse effects of dioxin-type compounds [8–10], the underlying mechanisms for this remain elusive. The putative functions of the previously identified target genes for AhR appear unlikely to fully explain the diverse range of biological actions of AhR ligands [11] (Fig. 1). The discovery of $CUL4B^{AhR}$ suggests that the adverse effects of AhR ligands in sex hormone signaling are, at least in part, attributable to the enhanced degradation of sex steroid receptors through E3 ubiquitin ligase activity of AhR [18,19] (Fig. 5). Target selectivity of the transcriptional regulatory system and the ubiquitin–proteasome system depends on specificity conferred by sequence-specific transcription factors and E3 ubiquitin ligases. To date, however, no single factor has been shown to function as a specificity factor in both target selection systems. Therefore, AhR is the first sequence-specific transcription factor identified that acts as an E3 ubiquitin ligase that also targets substrates for accelerated protein degradation. It is possible that other transcription factors, such as nuclear receptors, also function as E3 ubiquitin ligase components in some cellular contexts. Fat-soluble ligands for nuclear receptors are reported to have 'non-genomic' actions independent of transcriptional regulation-mediated effects. Considered together, ubiquitin ligase-based signaling mechanisms may possibly be involved in these non-genomic actions of various fat-soluble ligands.

From a mechanical point of view, AhR appears to be a unique and atypical type of substrate-specific component in cullin-based E3 complexes. AhR does not bear the reported signature motifs such as F-box [39], but directly associates with CUL4B. Substrate recognition by the other substrate-specific components in ubiquitin ligase complexes is usually evoked by substrate modifications such as phosphorylation [38–41] and hydroxylation [43,44,58]. However, recognition and subsequent ubiquitination of sex steroid receptors by AhR requires dioxin-type ligands, and does not occur following normal modifications of sex steroid receptors. Thus, it is plausible that activation of atypical E3 complexes may be a strategy of sensors for environmental stresses to respond to these stresses (Fig. 6). Supporting this, Hsp70 acts as an atypical substrate-specific adapter within the CHIP E3 complex in response to heat shock stress [63]. Hsp70 interacts with misfolded proteins and promotes their degradation. It later undergoes auto-catalytic degradation through CHIP [63]. In response to DNA damage, an atypical E3 complex alters the stability of TIP60, which in turn regulates ataxia-telangiectasia

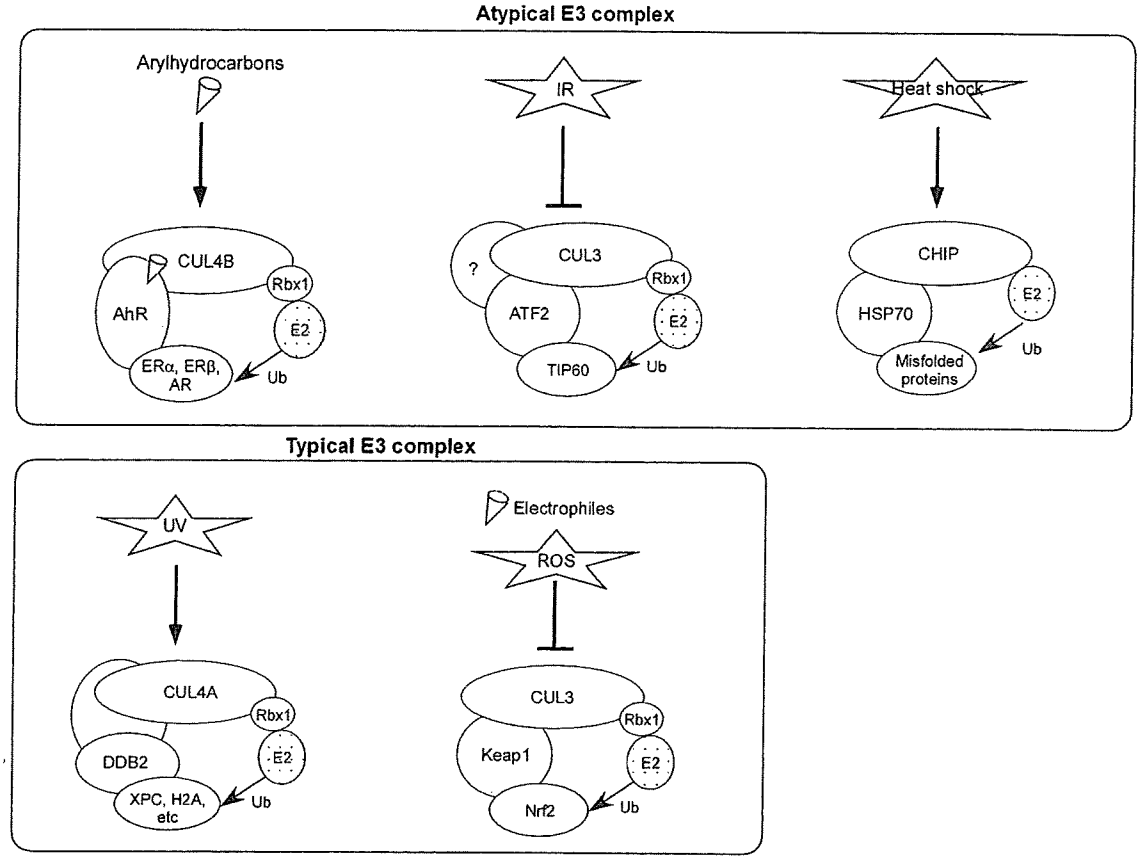

Fig. 6 – Atypical E3 complexes as sensors for environmental stresses. Several examples of E3 ubiquitin ligase-based perception of environmental stresses are illustrated. In the top panel, signal-responsive factors serve as atypical components of E3 complexes. In the bottom panel, canonical E3 components with conserved signature motif act as signal-responsive factors. ATF2, activating transcription factor-2; TIP60, tat interactive protein 60; CHIP, C-terminus of Hsp70 interacting protein; Hsp70, heat shock protein 70; XPC, xeroderma pigmentosum group C; H2A, histone H2A; Keap1, Kelch-like ECH-associated protein 1; Nrf2, NF-E2-related factor 2; IR, ionizing radiation; ROS, reactive oxygen species.

mutated (ATM) activation in DNA repair [64]. Activating transcription factor-2 (ATF2) promotes the degradation of TIP60 by assembling a CUL3-based complex under non-stressed conditions. ATF2 dissociates from TIP60 in response to ionizing radiation (IR), resulting in enhanced TIP60 stability and activity [64]. Functional regulation of E3 components is also seen with the CUL3-based component Keap1 in the oxidative stress response [65], and CUL4A-based components DDB2 and CSA in the DNA damage response [61]. Considered together, E3 components that respond to environmental stress may be more diverse than initially believed (Fig. 6). It is possible that $CUL4B^{AhR}$ may cross-talk with these stress-responsive E3 ligases to modulate their functions. As WDXR/DWD motif containing components, including DDB2 and CSA, also bind to CUL4B [46], it is possible that AhR may associate or interfere with these CRL subunits.

The E3 ubiquitin ligase activity of AhR and the transcriptional activity of AhR appear to be responsible for a distinct set of biological events induced by AhR ligands (Fig. 5). As substrate-specific adapters of ubiquitin ligase complexes are capable of recognizing a number of proteins, identification of other $CUL4B^{AhR}$ substrate proteins may reveal new molecular links between AhR-mediated signaling and other signaling pathways and cellular events. In this regard, it is of interest that AhR interacts with various transcription factors [11], such as Rb/E2F1 [66], SF1/Ad4BP [33], and NF-κB [67], to modulate their functions. AhR has recently been shown to regulate the differentiation of Th17 and T_{reg} cells [68–70]. This may be mediated by a functional interaction with STAT1 [70]. In addition, although the underlying mechanisms remain unknown, AhR also modulates the function of transcription factors [71] such as GR and RAR [72,73]. Considering the evolutionary conservation of AhR, it is likely that the intrinsic function of AhR is to mediate the signal transduction of endogenous ligands in cross-talk pathways. A current area of interest is the identification of candidate degradation substrates for AhR which are abnormally stabilized in AhR-deficient mice. In summary, several lines of recent evidence define a novel role for AhR as a ligand-dependent E3 ubiquitin ligase to regulate target-specific protein destruction. The ubiquitin ligase activity of AhR, together with the cross-talk of AhR with nuclear receptors through direct association, provides an additional layer of complexity for AhR biology. Characterization of these new molecular aspects of AhR function may lead to a greater understanding of the diverse biological actions induced by endogenous and exogenous AhR ligands.

Conflict of interest

The authors declare no competing financial interests.

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An Evaluation of Performance Standards and Non-radioactive Endpoints for the Local Lymph Node Assay

The Report and Recommendations of ECVAM Workshop 65[a]

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Preface

This is the report of the 65th of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM).

The main objective of ECVAM, as defined in 1993 by its Scientific Advisory Committee (ESAC), is to promote the scientific and regulatory acceptance of alternative methods which have scientific relevance and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures that would enable it to become well-informed about the state-of-the-art of non-animal test development and validation, and of opportunities for the possible incorporation of alternative methods into regulatory procedures. It was decided that this would be best achieved through a programme of ECVAM workshops, each addressing a specific topic, and at which selected groups of independent international experts would review the current status of various types of *in vitro* tests and their potential uses, and make recommendations about the best way forward.

A Workshop on *An Evaluation of Performance Standards and Non-radioactive Endpoints for the Local Lymph Node Assay* was held at ECVAM on 25–27 September 2007, under the chairmanship of David Basketter. The workshop was attended by experts from academia, industry, national organisations, and national and international validation authorities. At present, the local lymph node assay (LLNA) involves the use of radiolabelled thymidine as part of the standard protocol. The aim of the workshop was to review the status of methods which employ non-radioactive endpoints for the LLNA and to consider Performance Standards for their eventual assessment. At the end of the report are listed recommendations that should be considered for progressing toward the validation of relevant and reliable methods.

Key Definitions

To ensure the Performance Standards are applied appropriately, it is necessary to define their domain of applicability. For this purpose, the workshop participants debated in depth what could be considered to represent minor or major modifications to the standard LLNA. The following definitions were agreed:

Minor changes: those that maintain full compliance with OECD Test Guideline (TG) 429 (1), and that incorporate potential changes already foreseen in OECD TG 429. For a change to be considered minor, there is a requirement that the endpoint measured is still one of lymph node cell proliferation.

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[a]*This document represents the agreed report of the participants as individual scientists.*

Major changes: those that incorporate modifications to the standard LLNA that are broader in scope and of greater substance than those defined as being *minor*. Changes of this type would normally trigger a more thorough validation exercise, but should be considered on a case-by-case basis.

Introduction

The regulatory background

The approach used for the identification of chemicals with a significant degree of skin sensitisation potential is well characterised in the EU, and will soon be within the Globally Harmonised System (GHS; 2). The relevant European legislation includes the Dangerous Substances Directive, *Directive 67/548/EEC* (3), and the Dangerous Preparations Directive, *Directive 1999/45/EC* (4). With the advent of the legislation related to the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) system (5), further emphasis has been placed on the use of the most up-to-date methods, as well as ensuring that decisions are made by using all the available data, and with the minimum of additional animal testing. However, for confirmatory testing, the LLNA is the method of choice within the REACH system.

The tests traditionally used for the identification of chemicals possessing the intrinsic ability to cause skin sensitisation are the guinea-pig maximisation test (GPMT; 6), the Buehler occluded patch test (7) and the LLNA (8). The first two of these use a combination of the induction and elicitation phases in the guinea-pig, with the extent of sensitisation induction being determined as a function of the (erythematous) response to topical challenge. In contrast, the LLNA quantifies the induction response in mice by measuring proliferation in the lymph nodes which drain the site of topical application. The capacity of these methods to identify skin sensitisation hazard has only been formally validated for the LLNA (9–12). However, both within this validation process and via the publication of other datasets, the guinea-pig methods are also recognised to be of sufficient sensitivity and specificity (13–15).

For the purposes of hazard identification, skin sensitisation assays are interpreted in the same manner. In simple terms, if the results in the LLNA are positive (i.e. the stimulation of proliferation in test group lymph nodes is at least 3-times greater than in the concurrent vehicle-only-treated controls), or if at challenge ≥ 30% of the guinea-pigs are positive in a maximisation test, or if ≥ 15% of the guinea-pigs are positive in the Buehler test, then the substance is regarded as a skin sensitiser. The substance can then be classified formally and labelled, according to the EU system, as "R43: May cause sensitisation by skin contact". Thus, labelling can be applied to a chemical substance exclusively on the basis of data from a single animal test. Human experience can only be taken into account if it exists, and even then, it is normally not used to overturn the conclusion from positive animal data (16).

Ultimately, basic hazard identification is not sufficient for protection of human health; it merely represents the first step. Risk assessment and risk management are the processes that deliver human health protection. To permit this, some experts have proposed that, ideally, the relative potencies of skin sensitising chemicals should be determined and considered. The measurement of skin sensitisation potency has been the subject of much discussion in recent years, and expert groups in the EU (17), in European industry (18) and in the World Health Organisation (19) have made closely similar recommendations. Essentially, they all recommended that the optimal strategy is to determine the threshold positive concentration, the EC3 value, in the LLNA. It would not be appropriate to go into any detail of this measurement here, as it has been thoroughly reviewed elsewhere (20). However, what is important, is to appreciate its value for characterising skin sensitisation hazard and facilitating risk assessment (21, 22).

Background to performance standards

Prior to the acceptance of a new test method for regulatory testing, validation studies are conducted to assess its predictive capacity (the ability of the test method to correctly predict or measure the biological effect of interest, also referred to as accuracy) and its reliability (the extent of its intra-laboratory and inter-laboratory reproducibility). The LLNA underwent such a formal assessment before being adopted for use at the regulatory level. However, there might be cases for which a comprehensive validation exercise could be avoided and a simplified procedure applied. General criteria have been established by the validation authorities, and accepted at international level, to identify these cases and provide guidance for their assessment.

The concept of Performance Standards has been introduced as a way to streamline the validation process for test methods that are functionally and structurally similar to existing and adequately-validated test methods (23–25). As defined by the OECD (26), the purpose of Performance Standards is to communicate the basis by which new test methods, both proprietary (i.e. copyrighted, trademarked, registered) and non-proprietary, can be determined to have sufficient accuracy and reliability for specific testing purposes. These Performance