7-Ketocholesterol Is an Endogenous Modulator for the Arylhydrocarbon Receptor*

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We have identified 7-ketocholesterol (7-KC) as an endogenous modulator that inhibits transactivation by the arylhydrocarbon receptor (AhR) through competitive binding against xenobiotic ligands. 7-KC binds AhR and displaces labeled dioxin (2,3,7,8-tetrachlorodibenzo(α)pyrène (TCDD)). IC₅₀ is 5 x 10⁻⁷ M in vivo and 7 x 10⁻⁶ M in vitro. These figures are consistent with its concentration in human blood plasma and tissues. Association with 7-KC prevents AhR binding to DNA. 7-KC blocks the TCDD-mediated transactivation of stably expressed reporter gene constructs in T47-D cells as well as the expression of the endogenous CYP 1A1 gene in HepG2 cells and in primary porcine aortic endothelial cells. Injection of 7-KC to rats blocks the induction of CYP 1A1 messenger RNA and protein in endothelial cells from myocardial blood vessels. The differential sensitivity of mammalian species to toxic effects of AhR ligands, especially dioxin (TCDD), correlates with the expression of 7-KC from 7-hydroxycholesterol. The documented association with 7-KC from 7-hydroxycholesterol, which synthesizes 7-KC from 7-hydroxycholesterol, is expressed in large amounts in various tissues of the hamster, moderately expressed in humans and bovines, and is totally absent in rabbits, rats, and mice (8–10). This distribution closely correlates with the observed degrees of resistance to TCDD (reviewed in Ref. 1). Accordingly, by testing a panel of the most frequent oxysterols present in blood, we have identified 7-KC as an endogenous, physiological modulator for AhR. We describe here the parameters of this interaction and its inhibitory effects on the gene transactivation properties of the AhR in vitro and in vivo.

Extreme interspecies variations in toxicity are a striking feature of xenobiotics that bind the arylhydrocarbon receptor (AhR). Such compounds comprise dioxins (including the prototypical dioxin: 2,3,7,8-tetrachlorodibenzo(α)pyrene (TCDD)), benzo(α)pyrene (B(a)P), and polychlorinated biphenyls. A recent review discusses the numerous publications reporting that hamsters are very resistant to TCDD (LD₅₀ > 3 mg/kg), humans seem less sensitive than most laboratory animals, while guinea pigs, rats, and mice are most sensitive (LD₅₀ around 1 μg/kg), with detectable interstrain variations (1). This phenomenon is not restricted to AhR ligands: in most cases, the variable occurrence of a specific enzyme accounts for the variations in drug or hormone sensitivity (1). We recently described the binding of the trihydroxystilbene resveratrol to the AhR and the subsequent antagonization of toxic effects of AhR ligands (2). We observed the parallel between the alleged anti-inflammatory and cardioprotective role of resveratrol and the increasing amount of evidence for the involvement of AhR ligands in cardiovascular diseases (3–5). A variety of data suggest that these effects occur through their oxidative and atherogenic properties mediated by the AhR in the vascular endothelium (6, 7). Due to the structural requirements of the AhR for ligand accommodation, putative endogenous AhR ligand(s) or modulator(s) also involved in these mechanisms could be oxidized lipidic compound(s), preferably polycyclic hydrocarbons.

7-Hydroxycholesterol dehydrogenase (7-HCD), the enzyme that synthesizes 7-ketocholesterol (7-KC) from 7α,β-hydroxycholesterol, is expressed in large amounts in various tissues of the hamster, moderately expressed in humans and bovines, and is totally absent in rabbits, rats, and mice (8–10). This distribution closely correlates with the observed degrees of resistance to TCDD (reviewed in Ref. 1). Accordingly, by testing a panel of the most frequent oxysterols present in blood, we have identified 7-KC as an endogenous, physiological modulator for AhR. We describe here the parameters of this interaction and its inhibitory effects on the gene transactivation properties of the AhR in vitro and in vivo.

**EXPERIMENTAL PROCEDURES**

**Chemicals—** TCDD was a generous gift from Dr S. Safe (Texas A & M University, College Station, TX). 2,3,7,8-Tetrachloro(1,6-³H)dibenzo-p-dioxin, 28 Ci/mmol was purchased from Terrachem (Lenexa, KS). Dioxin stock solutions were initially dissolved in dimethyl sulfoxide and handled under a fume hood. TCDD stock was subsequently diluted in ethanol for use in experiments described below. Steroids were purchased from Steraloids (Wilton, NH). All other chemicals were purchased from Sigma.

**Cell Culture and CAT Assay—** All culture media were from Life Technologies, Inc. T47-D and HepG2 cells lines were routinely grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum, 1 gliter glucose, 2 mM sodium pyruvate, 50 mM sodium selenite, and 0.6 unit/ml (10⁻⁶ M) insulin. Unless stated otherwise, cells were established 24 h before any experiment in a modified form (stripped condition) of the medium: 1% charcoal-stripped newborn calf serum and 0.5 gliter glucose. All other components remained unchanged. The stable cell line 47DRE bearing the TCDD-responsive CAT construct was described in Ref. 2. Porcine aortic endothelial cells (PAEC) (a gift from Dr. Langille, Toronto General Hospital, Toronto, Ontario, Canada) of passage 4–7 were maintained in 199 medium supplemented with 5% fetal bovine serum.

**Northern Blot Experiments—** RNA from rat heart tissues were extracted using the TRizol reagent (Life Technologies, Inc.). 10 μg of RNA were electrophoresed on a 1% agarose-formaldehyde gel and transferred overnight to a nylon membrane (Nytran, Keene, NH). The membrane was dried at room temperature and then hybridized overnight at 60 °C with a ³²P-labeled rat CYP 1A1 cDNA probe. The blot was ex-
posed to a PhosphorImager screen and scanned using Image Quant software (version 5.0). The blot was later hybridized with rat β-actin cDNA, which served as a sample control. Scan generated values were used to calculate for each group the mean and standard error of the mean (S.E.) values.

**Western Blot Experiments**—Cells were treated with drugs in stripped condition as described in the figures legends and processed as described previously (2). The rabbit polyclonal antibody against CYP 1A1 and the CYP 1A1 protein standard were from Daichii Pure Chemicals Co. (Tokyo, Japan). The mouse monoclonal antibody against the AhR was from Affinity Bioreagents Inc. (Golden, CO). All were used at 1 μg/ml.

**Whole Cells Binding Assays**—T47-D cells were plated in six well culture dishes in standard passage conditions. At 70% confluence, cells were established in 2 ml of Opti-MEM I medium supplemented with 0.5 mg/ml bovine serum albumin for 24 h. Binding was performed the next day after a change of medium, in the CO2 incubator, at 37 °C for 20 min with labeled TCDD (5 nM) alone or challenged with unlabeled competitors. Cells were then taken at 4 °C and washed four times 10 min with 2 ml of phosphate-buffered saline (PBS) containing 0.5 mg/ml bovine serum albumin at 4 °C. Cells were lysed for 30 min at room temperature in 800 μl of cytosol buffer (20 mM Hepes, pH 7.6, 100 mM NaCl, 0.1 mM EDTA, 10% glycerol, 1 mM diethiothreitol, with Complete protease mixture (Roche Molecular Biochemicals, Meylan, France) containing 1% Nonidet P-40. Protein contents were measured, and aliquotes were counted for radioactivity: 500 μl of the supernatants were counted in 5 ml of Ultima Gold mixture (Packard, Meriden, CT) in a Beckman liquid scintillation counter (45% counting efficiency). Binding competition assays were repeated twice for each competitor and each point performed in duplicate.

**In Vitro Binding Assays**—Binding competition was performed using female New Zealand rabbit liver cytosol as the receptor source. The cytosol was prepared at 4 °C in 20 mM Hepes, pH 7.6, 1.5 mM EDTA, 10% glycerol, 10 mM β-mercaptoethanol, with Complete protease mixture (Roche Molecular Biochemicals, Meylan, France) by homogenization in an Ultra-Turrax homogenizer (Bioblock Scientific, Illkirch, France) followed with 20 strokes in a Dounce homogenizer with the tight pestle. The homogenate was centrifuged 30 min at 20,000 x g. The supernatant was centrifuged at 105,000 x g for 65 min. The cytosol was aliquoted, snap-frozen in liquid nitrogen, and kept at −80 °C. Cytosol aliquotes (4.5 ml) were thawed on ice, diluted 10-fold in 20 mM Hepes, pH 7.6, 0.1 mM EDTA, containing Complete protease mixture and 10 mM β-mercaptoethanol, and centrifuged 30 min at 105,000 x g. The cleared cytosol dilution was adjusted to 1 mM CaCl2 and 0.85 mg of protein/ml. 930 μl of diluted cytosol were preincubated with the desired amount of unlabeled competitors in ethanol solutions for 1 h at 4 °C. Subsequently, 2.3,7,8-tetrachloro1,6-6-Hdlibenzo-p-dioxin (28 Cl/mmol) was added at 0.2 nm and incubation continued for 3 h at 4 °C. Ethanol volume was adjusted in all tubes at 0.8% to optimize 7-KC solubilization without hampering specific binding. Nonplaceable binding was assessed by incubating the aliquots with 70 μl of a 2% activated charcoal suspension in 20 mM Hepes, pH 7.6, for 90 min at 4 °C, followed by centrifugation at 15,000 x g for 10 min. 500 μl of the supernatants were counted in 5 ml of Ultima Gold mixture (Packard, Meriden, CT) in a Beckman liquid scintillation counter (45% counting efficiency). Binding competition assays were repeated at least twice for each competitor, and each point was performed in triplicate.

**In Vitro DNA Binding**—T47-D cells were established in stripped conditions for 48 h. When they reached 90% confluence, they were treated with drugs for 90 min in the incubator, as described in the legend to Fig. 4. The probe used for gel retardation was a 35-base pair double-stranded oligonucleotide bearing a single copy of the CYP 1A1 legend to Fig. 4. The probe used for gel retardation was a 35-base pair double-stranded oligonucleotide bearing a single copy of the CYP 1A1 legend to Fig. 4. The probe used for gel retardation was a 35-base pair double-stranded oligonucleotide bearing a single copy of the CYP 1A1 legend to Fig. 4.

**Cell extracts** were prepared and gel retardation assays done as described previously (2). Another oligonucleotide was also used in chase experiments, bearing a copy of the sterol responsive element (SRE) from the low density lipoprotein receptor gene promoter (11): 5′-GATCCATT-TGAAATACCTACCCATGAAACTC-3′.

**In Vivo Antagonism Experiments**—36 male Harlan Sprague-Dawley rats were used for this 11-day study; group 1 were controls (n = 6). Animals were subcutaneously injected with olive oil (vehicle) on days 1, 3, 4, and 7. Group 2 were injected with 1 mg/kg B(a)P in DMBA on days 1 and 17. Group 3 (n = 10) were injected with 1 mg/kg B(a)P on days 1, 4, and 7. Group 4 (n = 10) were injected with mixtures of BioP/DMBA and 7-KC in a 1:1.1 ratio on days 1, 4 (7-KC only), and 7. Animals were sacrificed on day 11. Tissues were harvested and snap-frozen in liquid N2. Protein, mRNA, and immunocytochemical studies were then performed.

**Immunocytochemistry**—Rat tissue samples were fixed in phosphate-buffered parafomaldehyde (4%), dehydrated, and embedded in parafin. Paraffin sections were deparaffinized in xylene, rehydrated in alcohol series to water, and washed in PBS with 1% Triton X-100. Sections were incubated in 0.3% H2O2 for 30 min to quench endoperoxidase activity. Following PBS wash, sections were preincubated in 10% horse serum and 2% bovine serum albumin diluted in PBS for 30 min and then incubated with rabbit polyclonal antibody against CYP 1A1 at 1:500 overnight at 4 °C. Subsequent antibody detection was performed with biotinylated anti-rabbit mouse IgG and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA) with 3,3-diaminobenzidine as peroxidase substrate. Sections were then counterstained with hematoxylin, dehydrated, and mounted in DPX Mountant (Fruka, Toronto, Canada).

**Results**

In the search for AhR-modulating oxysterols, we focused primarily on 7-KC and on other molecular species identified in human plasma lipoproteins at concentrations compatible with efficient receptor interactions. We expected the active compound to behave as antagonists on TCDD-like effects such as CYP 1A1 or interleukin-16 overexpression as well as on TCDD-mediated transactivation in general. From a panel of nine oxysterols present in human plasma, including those able to inhibit TCDD-mediated transactivation, only 7-KC was able to displace labeled [3H]TCDD from the AhR in vivo whole cells uptake competition assays. Of those tested, only 7-KC was capable of displacing approximately 80% of the labeled TCDD from the receptor at 5 x 10⁻⁶ M. Briefly, T47-D cells were incubated with unlabeled oxysterols (5 x 10⁻⁶ M) and [3H]TCDD (5 x 10⁻⁹ M) for 20 min in a 37 °C CO2 incubator. Cells were extensively washed and lysed in a cytosolic buffer containing 1% Nonidet P-40. Protein aliquots were then quantitated and counted in a Beckman liquid scintillation counter. Assays were repeated twice, and each point was performed in triplicate. Mean ± S.E. are shown.

**7-Ketocholesterol Binds AhR**

Nine oxysterols present in human plasma were tested for their ability to displace labeled [3H]TCDD from the AhR in vivo whole cells uptake competition assays. Of those tested, only 7-KC was capable of displacing approximately 80% of the labeled TCDD from the receptor at 5 x 10⁻⁶ M. Briefly, T47-D cells were incubated with unlabeled oxysterols (5 x 10⁻⁶ M) and [3H]TCDD (5 x 10⁻⁹ M) for 20 min in a 37 °C CO2 incubator. Cells were extensively washed and lysed in a cytosolic buffer containing 1% Nonidet P-40. Protein aliquots were then quantitated and counted in a Beckman liquid scintillation counter. Assays were repeated twice, and each point was performed in triplicate. Mean ± S.E. are shown.
Squares or 7-KC (open circles). A, in vivo binding competition assay: 7-KC displaces labeled TCDD from the arylhydrocarbon receptor (AhR) of T47-D cells at physiological concentrations found in human blood plasma. The assay was performed as in Fig. 1. The IC_{50} for 7-KC competition of TCDD binding in vivo is 5 × 10^{-6} M. B, in vitro binding competition assay for rabbit liver cytosol AhR: 7-KC displaces labeled TCDD with an IC_{50} of 7 × 10^{-6} M. Specific binding in cytosol was 913 ± 35 dpm/mg protein (mean ± S.E.). Nonspecific binding amounted to 50% of total binding. With a specific activity of 4937 dpm/pmol, the specific binding corresponds to 183 ± 7 fmol of AhR per mg of rabbit liver cytosolic protein in these nonsaturating conditions (n = 6).

SRE was not directly amenable to gel retardation electrophoresis, probably due to the observed weaker affinity. Preincubation with a polyclonal antibody against AhR inhibited complex formation as previously shown (2–13).

We confirmed these in vitro data with endogenous CYP 1A1 expression experiments. Western blot experiments showed that 7-KC efficiently blocks the induction by TCDD of the endogenous CYP 1A1 protein in HepG2 cells (Fig. 5). When administered alone, 7-KC is able to suppress the basal level of CYP 1A1 expression observable in this cell line. This supports our hypothesis of 7-KC as a protective, inhibitory modulator of AhR. We also analyzed the inhibition of B[a]P-mediated induction of CYP 1A1 protein in primary cultures of PAEC by Western blot analysis to eliminate the possibility that our results could be limited to the above mentioned established cell lines. 7-KC efficiently reduced CYP 1A1 protein levels induced by B[a]P/DMBA in PAEC. In contrast, resveratrol was ineffective in PAEC (Fig. 6). This discrepancy could be due to a faster metabolism of resveratrol in these cells or to the inability of resveratrol to bind the porcine AhR.

Finally, we ascertained whether these results would concur with the in vivo situation. In keeping with our previous work (2) and to focus on the mechanism of tobacco-related cardiovascular diseases, rats were treated by subcutaneous injections of B[a]P/DMBA (1 mg/kg) alone or in conjunction with 7-KC (1 mg/kg) as described under “Experimental Procedures.” The ability of 7-KC to prevent B[a]P-mediated induction of CYP 1A1 was demonstrated by Northern blot followed by scanning densitometry of the bands (Fig. 7A), Western blot (Fig. 7B), and immunocytochemistry (Fig. 8). As can be seen on the photomicrographs in Fig. 8, CYP 1A1 induction by B[a]P/DMBA was limited to the endothelial cells of myocardial blood vessels and...
CYP 1A1

![Image](https://via.placeholder.com/150)

**Fig. 5.** 7-KC competes CYP 1A1 induction by TCDD in HepG2 cells. HepG2 cells were treated with ethanol (0.1%), TCDD (10⁻⁶ M), resveratrol (5 × 10⁻⁶ M), or 7-KC (5 × 10⁻⁶ M). Proteins were extracted from HepG2 cells following a 48-h treatment and separated by SDS-polyacrylamide gel electrophoresis. The resolved proteins were transferred to membrane and detected with a polyclonal antibody against CYP 1A1. As with resveratrol, 7-KC reduced the protein levels of CYP 1A1 induced by a 48-h treatment with TCDD. 7-KC alone elicited suppression of the basal levels of CYP 1A1 protein in this cell line.

![Image](https://via.placeholder.com/150)

**Fig. 6.** 7-KC reduces CYP 1A1 levels in PAEC induced by B(α)P/DMBA exposure. PAEC (a gift from Dr. Langille, Toronto General Hospital, Toronto, Ontario, Canada) at passages 4–7 were maintained in 199 medium supplemented with 5% fetal bovine serum. Cells were treated with B(α)P/DMBA (10⁻⁷ m) and 7-KC (in varying concentrations) and resveratrol (10⁻⁸ m) for 48 h. Protein extracts were harvested as described previously, and CYP 1A1 protein levels were examined by Western blot. As was observed in the rat, 7-KC reduced CYP 1A1 protein levels induced by B(α)P/DMBA. In contrast, resveratrol did not affect the protein levels of CYP 1A1 in this experiment. Lane 1, CYP 1A1 protein standard; lane 2, control (ethanol 0.1%); lane 3, B(α)P/DMBA; lane 4, B(α)P/DMBA and resveratrol; lane 5, resveratrol; lane 6, 7-KC; lane 7, B(α)P/DMBA and 7-KC (10⁻⁷ m); lane 8, B(α)P/DMBA and 7-KC (10⁻⁸ m); lane 9, B(α)P/DMBA and 7-KC (10⁻⁹ m).

absent from myocardial tissue. 7-KC efficiently suppressed the overexpression of CYP 1A1.

**DISCUSSION**

The search for a physiological, endogenous ligand for AhR has been ongoing for several years and has produced very dissimilar contenders such as lipoxin A₄, bilirubin, and tryptophan or vitamin B₆ metabolites (14–17). None of these compounds shed any light on the various features of AhR ligands toxicity and especially the interspecies variation in sensitivity to AhR ligands. Our observation, from literature analysis, that species-specific 7-HCD expression was correlated with TCDD resistance led us to describe this inhibitory interaction between 7-KC and the AhR.

The known examples of species-specific variation in sensitivity to drugs are linked to the variable occurrence of enzymes that either metabolize the xenobiotic (aflatoxin B₁ resistance of mice) or convert it into a more toxic compound (rapid acetylator phenotype for heterocyclic amines and generation of carcinogenic diol-epoxides from benzo(a)pyrene), as was reviewed in Ref. 20. In contrast, we present here a different mechanism, i.e., the production of an endogenous antagonist compound as a protective agent against xenobiotics. The possibility that AhR is redundant with other more recent lipid-dependent modulators such as adipocyte differentiation factor 1 (19) or oystersterol receptors (20) or represents a specialized subdomain in lipid metabolism remains open. The 7-HCD gene has not been described yet, nor has the regulation of its expression in 7-HCD-expressing mammals. In any case, it will be interesting to search for correlations between predisposition to certain cancers or diseases (especially cardiovascular diseases) and genetic polymorphism or mutations occurring in this gene.

Cholesterol and oxysterols are basic elements in cellular homeostasis for the constitution of membranes as well as steroidogenesis (reviewed in Refs. 21 and 22). It was initially believed that oxysterols were mainly cytotoxic molecules, but cytotoxicity assays for oxysterols were usually conducted with nonphysiological concentrations (10⁻⁴ to 10⁻³ m), utilizing rather crude end points (21, 22). However, as first proposed by Kandutsch and Chen (23), oxysterols are now more and more recognized as bona fide regulatory molecules in various physiological domains (reviewed in Ref. 24). The suspicion for cytotoxicity is now aimed at more oxidized molecules such as cholesterol peroxides (25, 26). 7-KC may be only a precursor of these more toxic molecules, synthesized in an uncontrolled way during cell culture experiments using high levels of 7-KC. However, this controversy is still fueled by a wealth of data on cytotoxic and apoptotic effects of 7-KC and other oxysterols (reviewed in Ref. 27). Oxysterols originate from the oxidation of dietary cholesterol but also from endogenous pathways involving 7-hydroxylases and 7-HCD (8–10). Their ability to repress enzymatic activities has been known for 20 years, but their direct impact on gene expression through the activation of nuclear receptors is a more recent concept. Receptors from the steroid superfamily existing as heterodimers with retinoid X receptor have been identified and shown to mediate transactivation either under the control of bile acids (FXR) or of a variety of hydroxysteroids substituted in 4, 7, 20, 22, or 25 (LXR) (reviewed in Ref. 20). These receptors have a low affinity for their ligands (Kₐ values around 10⁻⁶ to 10⁻⁵ m), but the values
are in the range of the blood plasma concentrations of these oxysterols. We were unable to assay a complete panel of 7-ketosteroids and 7-ketocholesterols, due to the lack of availability of many of them, especially derivatives of sex hormones. Nevertheless, most of these compounds are present in minute amounts and could hardly participate in a regulatory mechanism involving the AhR. 7-KC, on the contrary, is a major sterol constituent of HDLs (12). We were unable to achieve complete competition beyond 41% due to the lack of solubility of higher 7-KC concentrations. Setting up the assay revealed that efficient competition of TCDD binding to the AhR by 7-KC required preincubation of the competitor before addition of the labeled TCDD as well as the presence of 1 mM calcium chloride in the incubation medium. Such specific conditions, unnecessary for our classic assay (2), suggest that TCDD and 7-KC may have different structural requirements for binding to the AhR if not distinct binding sites. Hence our use of the word “modulator” instead of “endogenous ligand.” This hypothesis is currently under scrutiny in our laboratory. Other oxysterols reported in the Dzeletovic study (12) are present in similar concentrations in humans: 10−9 to 10−7 M for 7α- and 7β-hydroxycholesterol and 0 to 10−7 M for cholesterol epoxides and cholestanetriol (12). In contrast to 7-KC, these compounds are not known to be restricted to any particular mammalian species, and they do not bind the AhR. 7- and 6-ketocholestanes are also unable to bind. This high structural specificity of the interaction further supports the relevance of our findings. The physiological importance of our proposed interaction between AhR and 7-KC is considered at the double level of ligand preference and cross-talk between receptors. AhR has been shown to be able to counteract several members of the steroid receptor superfamily, such as the estrogen receptor (Ref. 39 and references within) or the progesterone receptor (2). As an example of the multiple levels of possible interplay, cholesterol 7-hydroxylases are induced by LXR activated by 22-OH cholesterol. This enzyme is the first step toward 7-KC biosynthesis. 7-KC will in turn repress the expression of these hydroxylases (reviewed in Refs. 21 and 22).

The involvement of AhR in human diseases can now be scrutinized under multiple new approaches.

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