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内分泌攪乱性確定試験としてのラット一生涯試験の試み

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Rat One-lifespan Test as a Definitive Test for Endocrine Disruptors

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In order to establish a definitive test protocol for endocrine disruptors, a one-lifespan test was performed using rats and the aging process of reproductive function was observed. Neonates of Sprague-Dawley rats received forced oral administration of diethylstilbestrol (DES) at doses of 0 (vehicle), 0.05, 0.5 and 5 μg/kg for 5 days after birth. Sexual maturation (vaginal opening and preputial separation), estrous cycles (from 8 to 49 weeks of age), mating (at 12, 23, 34, 56 and 68 weeks) and litter size (of the 1st to 3rd parturitions) were observed. Each half of the males were examined for sperm counts and organ weights at 26 and 52 weeks of age. In half of the females, hCG induced ovulation and organ weights were examined at 54 weeks of age. Then the observation of remaining animals was terminated at 101 weeks and survival rate were determined.

Vaginal opening in the group received DES at 5 μg/kg was significantly earlier than the vehicle control group. Normal estrous cycles were observed in no animals of 5 μg/kg DES group throughout the study, and in less than 10% of 0.5 μg/kg DES group at 28 weeks and on. Fertility rate of 12 week-old females of the 5 μg/kg DES group was 0%, and that of 23 week-old females of the 0.5 μg/kg group was 33.3%. Mating rate of 0.05 μg/kg females of this age was reduced to 60%. Influence of neonatal DES exposure was not observed in the first delivery in any group, but in the second parturitions litter size was reduced significantly in the 0.5 μg/kg group. Organ weights of 54 week-old females showed dose-related significant increase of pituitary weight in the 0.05 to 5 μg/kg groups. Adrenal weight was increased in the 0.5 and 5 μg/kg groups. Weight of ovaries was lowered significantly in the 0.5 and 5 μg/kg groups. Testing of induced ovulation with hCG revealed lack of influence of DES on number of shed oocytes. No effects of neonatal DES exposure in males were observed on preputial separation, fertility, sperm counts and organ weights. The lower survival rate was observed in the 5 μg/kg group females.

These results showed that early life exposure of low doses of DES potentially cause precocious sexual maturation, and decreases in reproductive function such as estrous cyclicity, fertility or litter size in female rats. These effects were considered to cause through disruption of hypothalamo-pituitary system, not through direct disturbance on ovarian function. The effects of DES observed in this study indicate the usefulness of one-lifespan test as a definitive test protocol for endocrine disruptors.

緒言

現在，内分泌攪乱化学物質（環境ホルモン，EDC）研究の焦点は，化学物質の内分泌攪乱性を確定する試験法の開発にある．ホルモン活性を有する化学物質が環境中にも存在することは既知の事実で，化学物質のホルモン活性の有無を検討する方法はEDCのスクリーニング試験となり得る．しかし，ホルモン活性を有する化学物質が，生体に有害な影響すなわち内分泌攪乱性を示すか否かを判定する試験法は確立されていない．実

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際，これら外因性ホルモン活性物質よりはるかに強力な受容体結合性を持つ内因性ホルモンの影響が，従来の生殖発生毒性試験（多世代繁殖試験）では確認されないにもかかわらず，ジエチルスチルベストロール（DES）のような物質では内分泌系など高次調節系の遅発性の異常が臨床的に起っている．つまりEDCは実際に存在し，それを試験する方法が求められている．このような理由から，現在，EDCの確定試験として従来の多世代繁殖試験に代る「一生涯試験」が考案された．

本研究ではエストロゲン活性を有するDESをSprague-Dawley（SD）ラットの新生児期に投与し，児の発達，成熟および老化に至る各段階において生殖器系機能の変化を検索する「ラット一生涯試験」を試みた．本研究では，新生児期DES投与が引き起こす遅発性の生殖機能異常を検索するために，雌は8週齢から49週齢まで性周期を観察し，12，23および34週齢で交配実験を行った．雄については，26および52週齢で精子検査を行い，12，23，34，56および68週齢で交配実験を行った．

材料および方法

試験には，日本チャールス・リバーから8週齢で入手したCrl:CD（SD）雌雄ラットを使用した．SD系ラットは，毒性試験において一般的に用いられている系統であり，生殖毒性に関する背景データが豊富で，Wistar系ラットに比べて性周期の加齢性変化が早期に起り易いことが知られている[1]．11週齢時に交配し，交尾が確認された雌を1群12匹以上からなる4群に振分けた．動物は温度22～25℃，湿度50～65%，照明12時間（7時～19時点灯）に調節された飼育室で，固型飼料（CE-2，日本クレア）と水道水を自由摂取させて飼育した．妊娠雌は，紙パルプ製チップを入れた金属製ケージに1匹ずつ収容した．全ての実験操作は，「財団法人食品薬品安全センター秦野研究所 動物実験に関する指針」に基づいて実施した．

EDCには子宮内あるいは新生児期の曝露での影響が指摘されていることから，DESの投与経路は新生児への強制経口投与を選択した．投与量は，内分泌攪乱化学物質に対する厚生労働省の試験スキーム[2]を考慮し，子宮肥大試験の結果をもとに設定した．すなわち，0.05～15 μg/kg/dayのDESを卵巣摘出マウスに3日間反復経口投与し，最終投与の約24時間後に子宮重量を測定した結果，5 μg/kg/day以上を投与した群で子宮重量が有意に増加したことから，5 μg/kg/dayを確実影響量として一生涯試験の最高用量に設定し，無影響量と考えられる0.5 および0.05 μg/kg/dayをそれぞれ中用量および低用量に設定した．投与液は，DES（Sigma-Aldrich, St. Louis, MO）20 mgを1 mLのエタノールに溶解し，コーン油で段階希釈して調製した．

新生児は，生後1日（分娩日を生後0日とする）に性別および外表奇形の有無を検査し，異常のない雌雄各5匹を1腹毎に選抜し，四肢の皮下に墨汁を注入して個体識別した．投与は生後1日から生後5日まで1日1回，マイクロシリンジおよび新生児用カテーテル[3]を用いて行い，投与液量は10 mL/kgとした．投与終了後は同腹児数を雌雄各4匹に調整し，生後21日に離乳させた．離乳後は，金属製金網床ケージに2匹ずつ収容した．体重は，生後0～5日（毎日），7，14および21日に測定し，離乳後は週1回，10週齢以降は隔週1回，26週齢以降は4週間毎に測定した．

雌は生後25日から膣開口を，雄は生後35日から陰茎包皮分離[4]を性成熟の指標として毎日観察した．各腹の雌2匹は，8週齢から49週齢まで2週間間隔で連日2週間，膣垢を採取し，性周期を観察した．膣垢像は発情前期，発情期，発情休止期に分類し，渡辺らの報告[1]と同様に性周期の型を分類した．各腹の雌雄各2匹は，12，23および34週齢から2週間を限度に，兄妹交配を避けて1：1で群内交配させた．群内交配で交尾が確認されなかった場合，雄は無処置雌と，雌は交尾が確認された同群の雄と，いずれも2週間を限度に再交配させた．雄は，さらに56および68週齢から2週間を限度に無処置雌と交配させた．群内交配で交尾が確認された雌は自然分娩させ，妊娠日数および産児数を確認し，哺育0および4日の哺育児体重を測定した．無処置雌は妊娠13日以降に帝王切開し，妊娠の有無を確認した．

雄は26，52および101週齢時，雌は54および

101週齢時にペントバルビタールナトリウム麻酔下で採血し，剖検した．雌雄とも101週齢以外の剖検時には，脳，下垂体，甲状腺，肝臓，脾臓，腎臓，副腎，精巣，精巣上体，前立腺（腹葉），精嚢（凝固腺を含む），卵巣，子宮の重量を測定した．

26および52週齢の剖検時に雄から採取し，凍結保存した精巣上体尾部および精巣を用いて精子数および精子頭部数を測定した．精巣上体尾部および精巣は，解凍後，ホモジナイズした精子懸濁液をModified IDENT STAIN Kit（Hamilton-Thorne）により染色し，HTM-IVOSにより，精巣上体尾部および精巣重量当たりの精子数および精子頭部数を求めた[5]．

各腹とも雌1匹は，54週齢時に排卵可能な卵胞の有無を確認するため，剖検16～17時間前にヒト絨毛性性腺刺激ホルモン（hCG，Sigma）を10 IU尾静脈内投与し，剖検時に卵管内の誘起排卵数を数えた[1]．

離乳前の児に関するデータは腹単位，離乳以降のデータは個体を標本単位として解析した．体重，器官重量，産児数および精子数のデータは，一元配置型の分散分析を行い，群間に有意差が認められた場合はDunnett法による多重比較検定を行った．性成熟，妊娠日数および生存率のデータは，Kruskal-Wallisの順位検定を行い，群間に有意差が認められた場合には，順位化した値を用いてDunnett法による多重比較を行った．交尾率および受胎率の差は，Fisherの直接確率法による検定を行った．有意水準は5％および1％とした．

結果

体重：生後0日から離乳まで，および離乳後から26週齢までの体重は，各群とも順調に増加し，雌雄ともDES投与の影響は認められなかった．また，26週齢以降の体重推移についても，46週齢から50週齢にかけて対照群の雌の体重が低下した以外に異常は認められなかった．

性成熟：雌の膣開口時期（平均±S.D.，日）は，5 μg/kg投与群（29.8 ± 2.2）で対照群（32.9 ± 1.7）より有意に早まったが，雄の陰茎包皮分離時期にはDES投与の影響はみられなかった．膣開口時期が早まった5 μg/kg投与群では，雌の全例で尿道開口部の過剰開裂[6]が観察された．

性周期：正常な性周期を示した雌の割合を図1に示した．5 μg/kg投与群では，観察を開始した8週齢から正常な性周期を示す雌は認められなかった．0.5 μg/kg投与群では8週齢から13週齢にかけては80％以上の雌が正常な性周期を示したが，20週齢から25週齢時には約50％，28週齢以降は10％未満となった．0.05 μg/kg投与群は対照

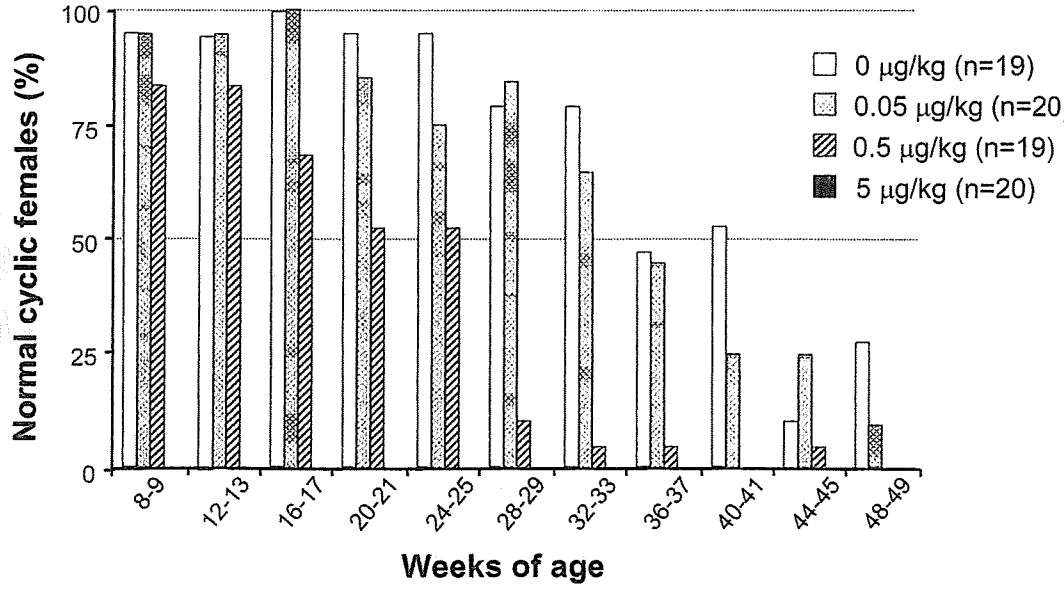

図1　新生児期にDESを投与したSD系雌ラットの性周期（正常性周期の割合の推移）

群とほぼ同様に推移し，正常な性周期を示す雌が29週齢までは80％以上，36週齢以降は50％未満となった．性周期を型別（図2）に見ると，5 μg/kg投与群で早期にみられた異常周期の型は連続発情であったのに対し，0.5 μg/kg以下の用量で加齢に伴って増加した異常周期の型は不規則周期や無発情であった．

交尾率・受胎率：交配結果を表1に示した．雄は12，23および34週齢のいずれの交配時期においても，交尾率および受胎率にDES投与の影響は認められなかった．また，56および68週齢の無処置雌との交配においても，DES投与の影響を示唆する変化は認められなかった．雌は12週齢の交配では，5 μg/kg投与群の交尾率は90％であったが，受胎率は0％となったため同群雌の23週齢以降の交配は中止した．23週齢の交配では，0.05 μg/kg投与群の交尾率が60％に低下し，0.5 μg/kg投与群の受胎率が33.3％に低下した．34週齢の交配では，対照群を含む各投与群の交尾率および受胎率が低下した．

分娩・哺育：分娩した雌の哺育成績を表2に示した．初回分娩では5 μg/kg投与群で産児が得られなかった以外にDES投与の影響は認められなかった．2産目では，0.5 μg/kg投与群の産児数が対照群より有意に減少し，0.05 μg/kg投与群の妊娠日数が対照群より延長する傾向にあった．3産目については，対照群を含む各投与群で受胎率が低下したことから，産児数の評価はできなかった．

雄の精子数および器官重量：26週齢および52週齢で精子数と器官重量を調べたが，いずれの時期においても，精巣上体尾部の精子数，精巣重量当りの精子頭部数，ならびに生殖器を含むいずれの器官重量にもDES投与の影響を示唆する変化は認められなかった．

雌の器官重量：54週齢の雌の器官重量を図3に示した．雌では，全てのDES投与群で下垂体重量が対照群より有意に増加し，5および0.5 μg/kg投与群で副腎重量が，5 μg/kg投与群で甲状腺重量が有意に増加した．また，5および0.5 μg/kg投与群で卵巣重量が対照群より有意に低下した．剖検時には，皮下に乳汁が貯留している例が0.05 μg/kg投与群で20例中2例，0.5 μg/kg投与群で18例中3例，5 μg/kg投与群で19例中9例みられた．その他，血中ホルモン濃度の測定では，0.05 μg/kg以上の投与群でプロラクチン濃度の上昇が，0.5 μg/kg以上の投与群でLH濃度の上昇

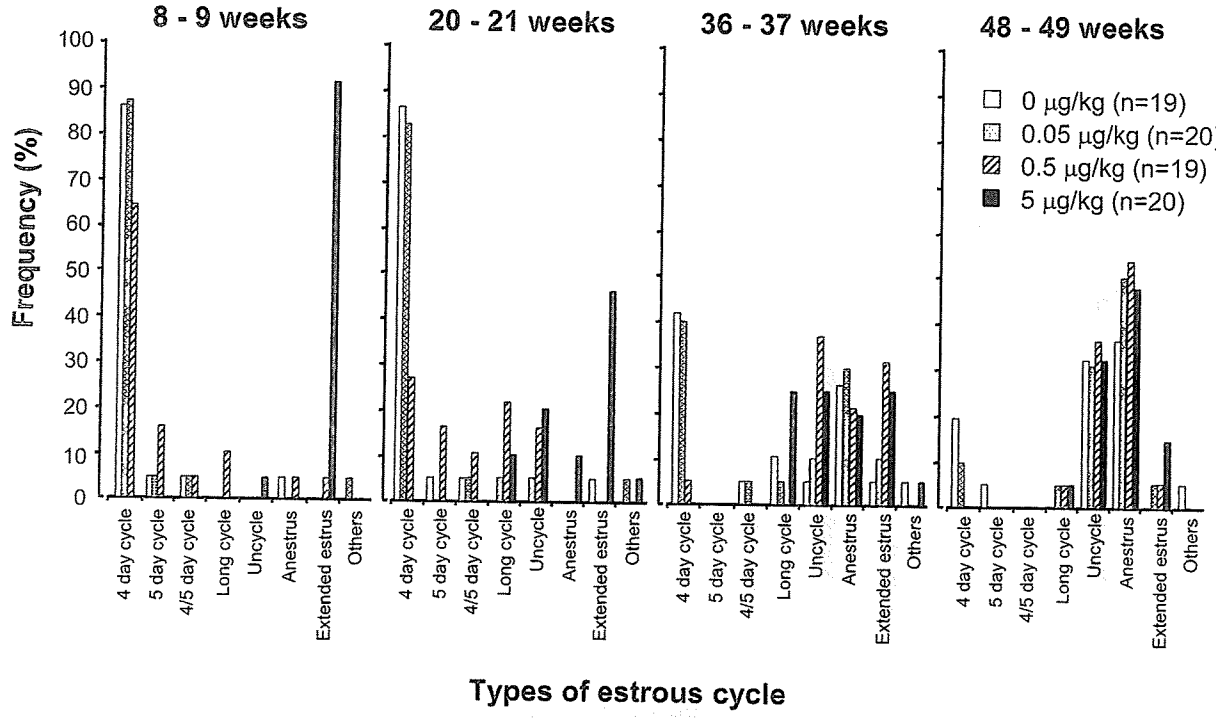

図2　新生児期にDESを投与したSD系雌ラットの性周期（性周期の型別の推移）

表1　新生児期にDESを投与したSD系ラットの交配成績

	Males				Females			
DES (μg/kg)	0	0.05	0.5	5	0	0.05	0.5	5
At 12 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	95.0 (19/20)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	95.0 (19/20)	100.0 (20/20)	90.0 (18/20)
Fertility index (%) (No. pregnant/no. copulated)	100.0 (20/20)	84.2 (16/19)	95.0 (19/20)	100.0 (18/18)	90.0 (18/20)	81.3 (13/16)	80.0 (16/20)	0.0 ** (0/18)
At 23 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	95.0 (17/20)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	60.0 ** (12/20)	90.0 (18/20)	
Fertility index (%) (No. pregnant/no. copulated)	89.5 (17/19)	100.0 (17/17)	100.0 (20/20)	83.3 (15/18)	80.0 (16/20)	58.3 (7/12)	33.3 ** (6/18)	
At 34 weeks of age								
Copulation index (%) (No. copulated/no. mated)	100.0 (20/20)	90.0 (18/20)	100.0 (20/20)	95.0 (19/20)	55.0 (11/20)	25.0 (5/20)	20.0 * (4/20)	
Fertility index (%) (No. pregnant/no. copulated)	95.0 (19/20)	72.2 (13/18)	90.0 (18/20)	84.2 (16/19)	54.5 (6/11)	60.0 (3/5)	25.0 (1/4)	
At 56 weeks of age								
Copulation index (%) (No. copulated/no. mated)	90.0 (18/20)	60.0 (12/20)	60.0 (12/20)	79.8 (15/20)				
Fertility index (%) (No. pregnant/no. copulated)	72.2 (13/18)	66.7 (8/12)	83.3 (10/12)	66.7 (10/15)				
At 68 weeks of age								
Copulation index (%) (No. copulated/no. mated)	57.9 (11/19)	47.4 (9/19)	55.6 (10/18)	55.6 (10/18)				
Fertility index (%) (No. pregnant/no. copulated)	72.7 (8/11)	66.7 (6/ 9)	80.0 (8/10)	50.0 (5/10)				

*, ** は対照群と比較して有意差（5%および1%）があることを示す.
データには無処置雌との交配結果も含まれる.

表2　新生児期にDESを投与したSD系母ラットの哺育成績

DES (μg/kg)	0	0.05	0.5	5
At the 1st parturition				
Number of dams	18	13	16	0
Gestation length in days	22.1 ± 0.3	22.2 ± 0.7	22.1 ± 0.5	
Number of newborns	13.9 ± 3.4	12.9 ± 3.9	14.1 ± 3.6	
Pup weight (g) Male	6.9 ± 0.3	6.8 ± 0.6	6.7 ± 0.7	
Female	6.5 ± 0.3	6.4 ± 0.6	6.3 ± 0.7	
Viability index on PND 4	99.7 ± 1.5	98.5 ± 3.0	99.0 ± 3.0	
At the 2nd parturition				
Number of dams	16	7	8	0
Gestation length in days	22.3 ± 0.5	22.9 ± 0.4 *	22.5 ± 0.5	
Number of newborns	12.8 ± 3.9	12.7 ± 3.7	7.6 ± 5.8 *	
Pup weight (g) Male	7.2 ± 0.8	6.9 ± 0.7	7.4 ± 0.9	
Female	6.7 ± 0.7	6.8 ± 0.7	6.6 ± 0.6	
Viability index on PND 4	93.3 ± 25.1	100.0 ± 0.0	100.0 ± 0.0	
At the 3rd parturition				
Number of dams	6	3	1	0
Gestation length in days	22.4 ± 0.5	22.7 ± 0.6	22.0	
Number of newborns	12.0 ± 4.3	11.0 ± 6.6	14.0	
Pup weight (g) Male	7.1 ± 0.5	7.2 ± 1.2	6.8	
Female	6.6 ± 0.5	6.8 ± 1.4	6.7	
Viability index on PND 4	96.5 ± 5.9	100.0 ± 0.0	100.0	

* は対照群と比較して有意差（5%）があることを示す. 各値は平均±標準偏差を示す.

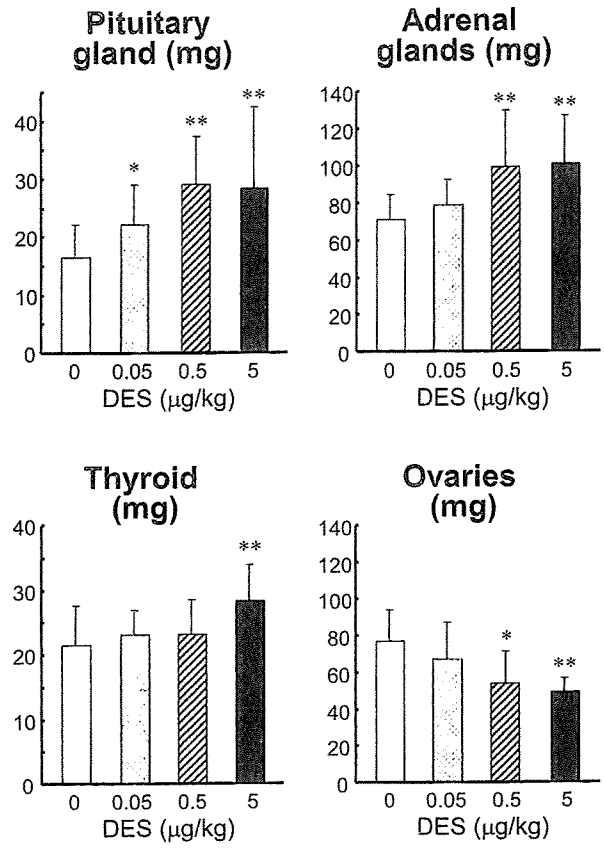

図3　新生児期にDESを投与したSD系雌ラットの54週齢における器官重量

*, ** は対照群と比較して有意差（5%および1%）があることを示す.

対照群（n=16），0.05 μg/kg群（n=20），0.5 μg/kg群（n=19），5 μg/kg群（n=19）

が確認されたが，T_3，T_4およびFSH濃度には，群間の差は認められなかった.

排卵検査：54週齢の排卵検査では，hCG投与により排卵した雌が対照群で8例中7例，0.05 μg/kg投与群で10例中6例，0.5 μg/kg投与群で10例中9例，5 μg/kg投与群で10例中8例みられ，誘起排卵数に群間の差は認められなかった.

生存曲線：雌雄の生存曲線を図4に示した．5 μg/kg投与群の雌では，生存日数が短縮したが，雄の生存日数にDES投与の影響はみられなかった.

考察

性成熟の観察では，5 μg/kg投与群で膣開口時期の早期化がみられ，同群では雌の尿道開口部の過剰開裂も認められた．性成熟の早期化[7]や尿道開口部の過剰開裂[6]を内分泌攪乱物質の生体に及ぼす有害影響と断定するには，さらに慎重に検討すべきであるが，一生涯試験の中では早期に検査できる項目であることから，後に得られる結果とあわせて内分泌攪乱性を判断する材料の一つになると考えられる．陰茎包皮分離時期に関しては，5 μg/kg投与群においても投与の影響はみられなかった．吉村ら[8]は，DESを出生後1～5日に投与したSD系ラットのうち，100 μg/kg以上の投与群で陰茎包皮分離時期の遅延を報告している．したがって，本研究で用いたDESの投与量では，雄の性成熟に影響を及ぼさないと判断される.

5 μg/kg投与群では8週齢から正常な性周期を示す雌動物は認められなかった．この結果から，同群ではDES投与により性成熟前の性腺刺激ホルモンが低下し，androgenizationを起し，膣開口後も排卵はなかったと推察される．一方，0.5 μg/kg投与群でも，16週齢以降に正常な性周期を示す動物の割合が減少した．TCDDの性成熟前投与[9]やビスフェノールAの胎生期投与[10]でも性周期の異常は対照群よりも早く起こることが示されている．これらのことは，内分泌攪乱化学物質の検索において，性周期を長期にわたって観察することの重要性を示している．本研究でみられた異常周期の型は，5 μg/kg投与群では主に連続発情であったのに対し，0.5 μg/kg投与群では不規則周期や無発情などが主であった．このことから，5 μg/kg投与群でみられた異常周期は無排卵に起因したのに対し，0.5 μg/kg投与群でみられた異常は，対照群にみられる加齢性変化が早期に誘発されたものと推察される.

12週齢の交配では，5 μg/kg投与群の雌で交尾が確認されたものの，受胎率は0%であった．これは，前述の排卵を伴わない連続発情を反映した結果と考えられる．一方，0.5 μg/kg投与群の雌では，23週齢の交配で受胎率が低下し，2産目の産児数が減少した．この変化も，同群で早期に加齢性変化，すなわち性周期の乱れを来したことと一致すると考えられ，2産目の産児数の低下は，エストロゲン分泌の低下に起因した排卵数の減少が原因と推定される[11]．なお，23週齢の交配で0.05 μg/kg投与群にみられた交尾率低下の原因は

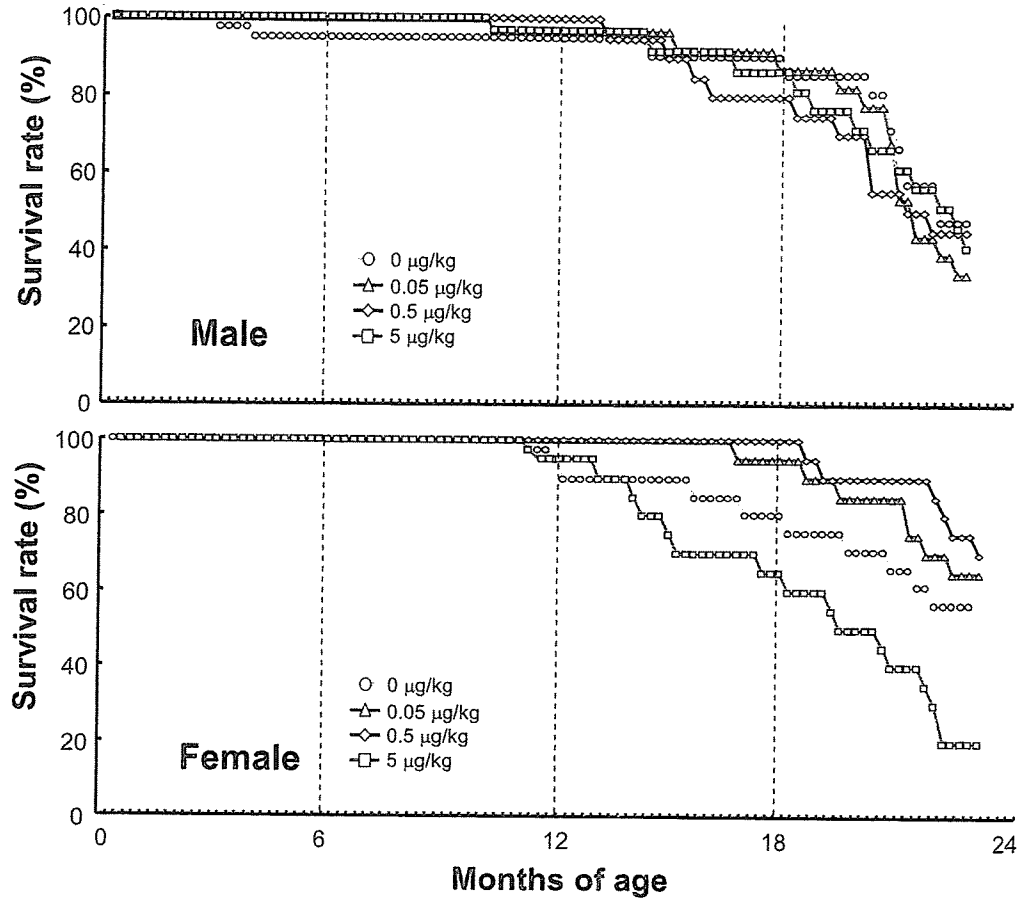

図4　新生児期にDESを投与したSD系ラットの生存曲線

不明である．

雄では，いずれの時期の交配においても，DES投与の影響は認められなかった．また，26週齢および52週齢では，ともに精子数および生殖器重量にDES投与の影響を示唆する変化も認められなかった．新生児期のDES投与では，雄動物の生殖能力への影響[12,13]が報告されているが，いずれも高用量（0.1 mg/pup/day以上）での報告であり，今回用いた5 μg/kgまでのDESは，新生児期投与で雄の生殖機能に影響を及ぼさないと考えられる．しかしながら，Vom Saalら[14]は，胎生期に0.02 μg/kgのDESを投与したマウスで前立腺重量の増加を報告していることから，同程度の投与量を用いて胎生期曝露を追試することが必要であろう．

54週齢の雌では，下垂体および副腎重量が用量に依存して増加し，血中プロラクチンとLH濃度の上昇が確認された．剖検時に乳汁貯留も観察されていることから，高プロラクチン血症が疑われる．一方，5 μg/kg投与群では甲状腺重量の増加が認められたが，血中T_3およびT_4濃度に変化はなかった．卵巣重量は，0.5 μg/kg以上の投与群で低下したが，hCG投与による誘起排卵がすべての投与群で確認されたことから，新生時期のDES投与は，卵巣機能には直接影響を及ぼさず，下垂体からの刺激の低下，すなわち視床下部－下垂体系の内分泌攪乱作用に起因したものと推定される．

結論

低用量DESは雌の性成熟を早めるだけでなく，雌の老化過程における性周期，受胎率，交尾率あるいは産児数に影響を与える可能性が示唆された．また，それらの変化は視床下部－下垂体系の内分泌攪乱作用に起因したものと推定され，卵巣機能への直接的な影響ではないと考えられた．本研究から，内分泌攪乱性を確定する上で一生涯試

験は有効であることが示されたが，雄の生殖機能異常を検出することは出来なかった．

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Anti-Androgenic Activity of *N*-Nitrosodibenzylamine, *N*-Nitrosodiphenylamine and *N*-Nitrosodicyclohexylamine

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When 56 selected environmental chemicals were tested for the androgenic activity to Yeast Two-hybrid and reporter gene assay in the presence of 5α-dihydrotestosterone (DHT), the activity was inhibited by some of the chemicals including *N*-nitrosodiphenylamine (NDPA), a novel anti-androgenic compound, and one of suspected carcinogenic *N*-nitrosocompounds (NOCs) commonly used as material of rubber and plastic goods. We further examined 15 NOCs for anti-androgenic activity, and found that *N*-nitrosodibenzylamine (NDBzA) and *N*-nitrosodicyclohexylamine (NDCHA) as well as NDPA inhibited the activity of DHT in a dose-dependent manner. These compounds showed the competitive binding to androgen receptor (AR) against DHT and decreased the level of AR protein. Furthermore, 3 NOCs down-regulated the prostate specific antigen (PSA) at the transcriptional level in LNCaP cells. These results suggest that some NOCs antagonized the androgenic effect of DHT in the same manner as the synthetic anti-androgen, flutamide (F).

Key words —— anti-androgenic activity, androgen receptor, *N*-nitrosocompound, *N*-nitrosodibenzylamine, *N*-nitrosodiphenylamine, *N*-nitrosodicyclohexylamine

INTRODUCTION

The androgens, testosterone (T) and its metabolite 5α-dihydrotestosterone (DHT), play an important role in the development and function of male reproductive organs such as prostate and testis, as well as non-reproductive organs including muscle, hair follicles and brain. Their biological effects are mediated by one of the nuclear receptor superfamily of ligand-regulated transcription factors, androgen receptor (AR).[1,2] T is synthesized mainly in the Leydig cells of testes and converted in the prostate to DHT, a more potent androgen than T. Upon DHT binding to AR in the cytosol, the complex translocates to the nucleus, where AR-DHT complex binds to androgen response element (ARE) in the promoter region of target genes and regulate the transcription of them.[3,4] The androgen target gene, a member of the human kallikrein gene family, produces prostate specific antigen (PSA), which is well known as a marker protein of prostate cancer.[5]

It has been noticed that some environmental and industrial chemicals interfere with endogenous androgen function in humans and wildlife. These compounds are referred to as endocrine disruptors (EDs). Interference with androgenic action can occur in a various developmental and reproductive abnormalities of the male sex functions.[6] Although there have been many reports on EDs, most of them are estrogenic action via estrogen receptor (ER). We therefore have been focused on anti-androgenic compounds, showing female phenotype via AR.

There have been many studies of screening for EDs by *in vitro* assays, such as Yeast Two-hybrid, reporter gene, and receptor binding assay.[7–10] Environmental anti-androgens, such as *p,p'*-dichlorodiphenyldichloroethylene (DDE), vinclozolin and linuron, compete with endogenous androgens for AR, to alter androgen-dependent transcriptions by inhibition of binding to AR.[11–16] Now more than

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50000 chemicals are distributed in the world, among which novel and potent anti-androgenic chemicals may exist. We should therefore assess androgenic action of these chemicals before they affect to humans and wildlife.

In this study, we first tested anti-androgenic activity of a total of 56 environmental chemicals by Yeast Two-hybrid and AR-EcoScreen cell reporter gene assay. These were performed flutamide (F) and hydroxyflutamide (HF) as a positive compound. F is well known as a synthetic anti-androgen and used for drug therapy of prostate cancer.[17] HF is an active metabolite of F.

We found a novel anti-androgenic compound, *N*-nitrosodiphenylamine (NDPA). *N*-Nitroso compounds (NOCs) including NDPA are well known to have carcinogenic and mutagenic properties, such that gastric, esophageal, nasopharyngeal, bladder and colon cancers.[18,19] However there have been no reports that NOCs affect endocrine systems. Exposure to environmental NOCs is through various pathways, for example, life-style (tobacco, food, cosmetic products and household commodities), occupational (rubber, leather, and material industry) and uptake of precursors (nitrite, nitrate and amine).[20] Thus it is thought that humans and wildlife have chance affected by NOCs. Then we tested anti-androgenic activity of 15 NOCs by using Yeast Two-hybrid and reporter gene assay. Finally we investigated the mechanism of anti-androgenic action of positive compounds.

MATERIALS AND METHODS

Chemicals and Cells —— All chemicals of the highest grade commercially available were used without further purification. Most of 56 test chemicals listed in Table 1 are the same used in the previous paper.[7] NOCs listed in Table 2 were purchased from Wako Pure chemicals (Osaka, Japan) and dissolved in dimethyl sulfoxide (DMSO) for use. AR EcoScreen cells were grown in 10 cm dishes using DMEM/F-12 (GIBCO, BRL, Inc., U.K.) supplemented with 5% heat-inactivated fetal bovine serum (FBS, ICN Biomedical, Inc., Aurora, Ohio), penicillin (100 U/ml), streptomycin (100 μg/ml) (Nakarai Tesque Co., Kyoto, Japan) in a humidified 5% CO_2 incubator. LNCaP cells, the androgen-sensitive human prostate cancer cell line, were cultured in 10 cm dishes using RPMI 1640 (Nacalai Tesque Co., Kyoto, Japan) supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 μg/ml) (Nacalai Tesque Co., Kyoto, Japan) in a humidified 5% CO_2 incubator.

Yeast Two-Hybrid Assay (AR:SRC-1) —— The Yeast Two-hybrid assay system with the rat AR and the coactivator, steroid receptor coactivator-1 (SRC-1), was prepared by modifying the method described in previous reports.[7,21] Briefly, two expression plasmids, pGBT9-AR-LBD and pACT2-SRC-1, were transformed into yeast cells (Saccharomyces cerevisiae Y190). The yeast cells (100 μl), pre-incubated overnight at 30°C in synthetic defined (SD) medium free from tryptophan and leucine, were incubated with NOCs (2.5 μl) and DHT (40 nM final concentration) in SD medium lacking tryptophan and leucine (150 μl) at 30°C for 4 hr. After the absorbance at 595 nm was measured, the cultured cells were digested enzymatically with zymolyase 20T (Seikagaku Co., Tokyo, Japan) at 37°C for 15 min. Then the lysate was mixed with 40 μl of *o*-nitrophenyl-β-D-galactopyranoside (4 mg/ml in Z-buffer) and incubated at 37°C for 1 hr. Finally, added 100 μl of 1 M Na_2CO_3 to stop the reaction and then absorbance at 420 and 570 nm were measured by using a 96-well microplate reader (Model 550 MICROPLATE READER, BIO RAD) and β-galactosidase activity was calculated from these 3 absorbances. The anti-androgenic activity was expressed as the percentage against β-galactosidase activity of 40 nM DHT without chemicals (100%). It was judged to be positive when the inhibition was more than 20% and the cytotoxicity was not observed at these concentrations. Cytotoxicity of the compound was confirmed by using control yeast cells which transformed pGBT9-p53 and pGAD3F-SV40 into yeast cells. IC_{50} values were calculated using GraphPad Prism 2.01 software.

Reporter Gene Assay for AR (AR-EcoScreen) —— The reporter gene assay using AR-EcoScreen cells was performed as previously described.[22] AR EcoScreen can evaluate androgenic activity and toxicity of compound. Briefly, in 24 well plates, AR-EcoScreen cells were seeded 1×10^5 cells/ml in phenol red free DMEM/F12 containing 5% charcoal-dextran treated fetal bovine serum (FBS). After 24 hr of culturing, medium was changed and added NOCs with 0.5 nM DHT. Following 16–24 hr of culturing, cells were washed twice with phosphate buffered saline (PBS), lysed with Passive Lysis Buffer (Promega Co., WI, U.S.A.) and assayed using Dual luciferase assay system (Promega Co., WI, U.S.A.) with luminometer (Lumat LB9501, Berthold

Table 1. Names of 56 Test Chemicals and Anti-Androgenic Activity in the Yeast Two-Hybrid Assay (AR:SRC-1)

Group, compounds[a]	
Pesticides and related (21)	Benzens and heterocyclics (9)
1,2-Dibromo-3-chloropropane	2,4-Dinitroaniline
2,4,5-Trichlorophenol	2,5-Dinitroaniline
2,4-Dichlorophenoxyacetic acid	2-Phenylendiamine
2,4,5-Trichlorophenoxyacetic acid	4-Chloroaniline
Alachlor[b]	Benzophenone[b]
Aldicarb	Biphenyl[b]
Captan	*N*-Ethylaniline
Carbaryl (NAC)[b]	4-Nitrotoluene
γ-Hexachlorocyclohexane (γ-HCH)[b]	*N*-Nitrosodiphenylamine (NDPA)[b]
Hexachlorophene[b]	Phthalates and adipate (9)
Maneb	Di-*n*-ethyl phthalate[b]
Manzeb	Di-*n*-propyl phthalate[b]
Methomyl	Di-*n*-butyl phthalate[b]
Methoxychlor (MXC)	Di-*n*-pentyl phthalate
Molinate[b]	Di-*n*-hexyl phthalate
Pentachlorophenol	Butylbenzyl phthalate[b]
Thiobencarb[b]	2-Ethylhexyl phthalate
Thiuram	2-Cyclohexyl phthalate
Vinclozolin[b]	2-Ethylhexyl adipate
Simazine	Aliphatics (4)
Ziram	Cyclohexyl amine
Phenols (9)	*N,N*-Dimethylformamide
2,4-Dichlorophenol	Nitrilotriacetic acid
2,4-Dinitrophenol	*N*-Nitrosodimethylamine
2,4,6-Tribromophenol	Flavonoids (4)
2,5-Dichlorophenol	Coumestrol
4-Cresol	Daizein
4-Nonylphenol[b]	Genistein
Bisphenol A[b]	Naringenin
Diethylstilbesterol (DES)	
N-Phenyl-1-naphthylamine[b]	

a) Compounds marked by *b*) were positive in Yeast Two-hybrid assay (AR:SRC-1).

GmbH & Co.). The anti-androgenic activity was expressed as the percentage against 0.5 nM DHT without chemicals (100%). We judged as anti-androgen when 20% or more inhibition was calculated without cytotoxicities. IC_{50} values were calculated using GraphPad Prism 2.01 software.

Competitive Binding Assay for AR —— The binding affinity of NOCs to AR was determined by a fluorescene polarization assay using ANDROGEN RECEPTOR COMPETITOR, GREEN Kit (Pan Vera, Madison, U.S.A.). Briefly, 1 μl of NOC/DMSO solution was added to 49 μl of AL green assay buffer in the small test tube. Additionally, added 50 μl of AR-ligand binding domain (LBD) (25 nM) / Fluormone AL green (1 nM) complex to the same tube and mixed. The assay tube covered to protect the reagents from light was incubated at 22°C for 5 hr. Finally, sample fluorescence was measured on BEACON 2000 (Pan Vera, Madison, U.S.A.). DMSO (0% inhibition) instead of the compound solution was used as a negative control, and 0.5 μl of Fluormone AL green (1 nM) instead of AR-LBD/Fluormone AL green complex as a positive control (100% inhibition). IC_{50} values were calculated using GraphPad Prism 2.01 software.

Protein Preparation and Western Blotting —— LNCaP cells were seeded at 2.0×10^5 cells/ml in RPMI 1640 medium supplemented with 10% char-

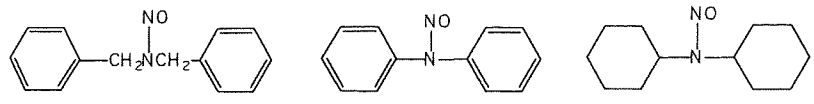
Table 2. Anti-Androgenic Activity of 17 Environmental Chemicals by Yeast Two-Hybrid Assay and Reporter Gene Assay

Group, compounds	Yeast Two-hybrid assay IC_{20} [a)]	Reporter gene assay IC_{20} [b)]
Pesticides and related		
Alachlor	+	++
Carbaryl	++	–
Hexachlorophene	++	–
γ-Hexachlorocyclohexane	+	–
Molinate	+	–
Thiobencarb	++	++
Vinclozolin	++	+++
Phenols		
4-Nonylphenol	+++	++
Bisphenol A	+	++
N-Phenyl-1-naphtylamine	+	–
Benzens and heterocyclics		
Benzophenone	+	–
Biphenyl	++	–
N-Nitrosodiphenylamine (NDPA)	++	++
Phthalates		
Di-*n*-ethyl phthalate	+	–
Di-*n*-propyl phthalate	++	+++
Di-*n*- butyl phthalate	++	++
Butylbenzyl phthalate	+	+++

a) Concentration of the test compounds showing 20% inhibition of the androgenic activity induced by 40 nM DHT. *b*) Concentration of the test compounds showing 20% inhibition of the androgenic activity induced by 0.5 nM DHT. Symbols: +++, anti-androgenic activity ($IC_{20} < 1$ μM); ++, anti-androgenic activity (1 μM $\leq IC_{20} <$ 10 μM); +, anti-androgenic activity (10 μM $\leq IC_{20}$); –, no effect.

coal-stripped FBS. After 24 hr of incubating, the cells in fresh medium were incubated for 10 hr with NOCs in the presence of 10 nM DHT. After the treatment, the cells were collected Passive Lysis Buffer (Promega Co., WI, U.S.A.) and centrifuged for 5 min. The supernatant was collected as a sample of Western blotting. 15 μg aliquots were separated by SDS-PAGE (7.5% acrylamide gel) and transferred to poly(vinylidene fluoride) (PVDF) membrane. The membrane was probed with rabbit anti-androgen receptor antibody (Upstate Biotechnology, Lake Placid, NY, U.S.A.), followed by peroxidase-conjugated anti-rabbit IgG antibody (Amersham Biosciences, Piscataway, NJ, U.S.A.). The membrane was then visualized using an electrochemical luminescence (ECL) detection system.

RNA Preparation and Northern Blotting —— LNCaP cells were seeded at 1.5×10^5 cells/ml in RPMI 1640 medium supplemented with 10% charcoal-stripped FBS. After 24 hr incubation, the medium was changed and NOC was added with 10 nM DHT. After 18 hr treated, total RNA was isolated using TRIzol reagent (Invitrogen Corp., Carlsbad, CA, U.S.A.). Total RNA (12 μg) was denatured in 50% formamide and 17.5% formaldehyde at 65°C and fractionated by electrophoresis on a 1% agarose gel containing 18% formaldehyde. Samples were transferred to nylon membrane (Hybond N^+, Amersham Life Sciences, Little Chalfont, Buckinghamshire, U.K.) in 20 × SSC (1 × SSC is 0.15 M NaCl and 0.0015 M sodium citrate). The DNA probes for PSA and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were generated from PCR products. PCR primers used for PSA (418-939 bp) were: forward; 5′-GGCAGGTGCTTGTAGCCTCTC-3′, reverse; 5′-CACCCGAGCAGGTGCTTTTGC-3′, and for GAPDH: forward; 5′-ACCACAGTCCATGCCATCA-3′, reverse; 5′-TCCACCACCCTGTTGCTGTA-3′. These products were labeled with [α-^{32}P]dCTP using the BcaBEST™ Labeling Kit (TaKaRa Bio. Inc., Ohtsu, Japan). Hybridization was performed overnight at 65°C in 7% sodium dodecyl sulfate (SDS), 1% bovine serum albumin (BSA), 137 mM Na_2HPO_4



NDBzA NDPA NDCHA

Fig. 1. Chemical Structures of 3 NOCs Showing Anti-Androgenic Activity

and 63.2 mM NaH_2PO_4.

Scanning Bio-Imaging Analysis —— Scanning bio-imaging analysis was performed with a BAS-2500 (FUJI PHTO FILM Co. LTD., Kanagawa, Japan). The area of PSA was integrated by GAPDH.

Statistics —— All results are expressed as means ± standard deviations (S.D.). Statistical analysis was performed by Dunnett's method.

RESULTS

Screening for Anti-Androgenic activity of the 56 Environmental Chemicals by Yeast Two-Hybrid Assay (AR:SRC-1) and AR-EcoScreen Cell Reporter Gene Assay

For anti-androgenic activity, the test chemicals were examined in the presence of 40 and 0.5 nM DHT in Yeast Two-hybrid assay (AR:SRC-1) and AR-EcoScreen cell assay, respectively. The 2 concentrations of DHT in the assay were corresponding to 50% and 70% of the maximum activity in each assay, and it was judged to be positive for the chemicals having IC_{20} values of lower than 10 μm. Names of the 56 chemicals tested are listed in Table 1, and positive in the Yeast Two-hybrid assay were marked by *.

As seen in Table 1, about one third, 17 of the 56 chemicals, were positive in the Yeast Two-hybrid assay. When these positive compounds were applied to the cell assay, 9 of them were positive in the reporter gene assay (Table 2). The result shows that 6 (thiobencarb, vinclozolin, 4-nonylphenol, NDPA, di-n-proyl phthalate, and di-n-butyl phthalate) were agreed in both assays, but the remaining were disagreed. Except for NDPA and di-n-proyl phthalate, their anti-androgenic activities had already been reported.

Screening for Anti-Androgenic Activity on 15 NOCs

In the last section, we found that NDPA and di-n-propyl phthalate were only newly found anti-androgenic compounds. Because anti-androgenic activities of the other phthalates had been tested, we focused on the anti-androgenic activity of NDPA, and NOC with testing 15 chemicals by two *in vitro* assays. Although no androgenic activity was observed on the 15 NOCs (data not shown), 3 NOCs [*N*-nitrosodibenzylamine (NDBzA), NDPA and *N*-nitrosodicyclohexylamine (NDCHA)] shown in Fig. 1 indicated significant and dose-dependent anti-androgenic activities by two *in vitro* assays (Table 3, Fig. 2, 3). IC_{50} values of them were 3, 28, and 55 μm in the Yeast Two-hybrid assay and 5, 17 and 12 μm in the AR-EcoScreen cell reporter gene assay, whereas IC_{50} of F was 5 and 0.2 μm, respectively (Table 4). Thus anti-androgenic activity of NDBzA was thought to be near F, whereas that of NDPA and NDCHA was about 10 times lower than these compounds.

Mode of Action of NDBzA, NDPA and NDCHA

Many anti-androgens inhibited androgenic action by competition on binding to AR. As shown in Fig. 4, NDBzA, NDPA and NDCHA showed the binding affinity to AR by the competitive binding assay using fluorescent labeling AR ligand. IC_{50} values of NDBzA, NDPA, NDCHA, F and DHT were 20, 183, 27, 29 and 0.04 *m*m, respectively (Table 4). And the binding affinity of NDBzA and NDCHA was estimated to be similar to F, and NDPA was about 10 times lower, although these affinities were 1000 to 10000 times lower than DHT.

The effect of these NOCs on the level of AR protein expression was examined in androgen dependent LNCaP cells. When the cells were treated with DHT for 10 hr, the level of AR increased. Then when LNCaP cells were treated with NDBzA, NDPA, NDCHA and F in the presence of DHT, they decreased the level of AR induced with DHT (Fig. 5). These results suggest that NDBzA, NDPA and NDCHA prevented the DHT induced AR level to inhibit the androgenic action of DHT and another pathway.

Northern blot analysis was applied to determine effect of NOCs on the expression of an endogenous androgen responsive gene in LNCaP cells. The level

Table 3. Effect of NOCs on Luciferase Activity

Compound	Relative β-galactosidase activity (%)	Relative luciferase activity[a] (%)	Source
N-Nitrosodimethylamine	95.7 ± 4.2	105.1 ± 13.9	Wako
N-Nitrosodiethylamine	103.6 ± 3.1	105.8 ± 9.5	Wako
N-Nitrosodipropylamine	108.3 ± 2.4	94.4 ± 9.1	SUPELCO
N-Nitrosodibutylamine	103.6 ± 2.9	95.8 ± 10.5	SIGMA
N-Nitrosodiisobutylamine	106.4 ± 1.7	95.3 ± 6.0	Wako
N-Nitrosomethylbutylamine	102.1 ± 4.5	102.8 ± 5.7	SIGMA
N-Nitrosoethylbutylamine	101.0 ± 2.9	99.5 ± 8.1	SIGMA
N-Nitrosodiethanolamine	113.4 ± 5.6	98.0 ± 8.4	SIGMA
N-Nitrosodiisopropanolamine	109.5 ± 2.8	91.7 ± 5.1	SIGMA
N-Nitrosodicyclohexylamine	50.7 ± 8.9**	54.2 ± 3.3**	SIGMA-ALDRICH
N-Nitrosodiphenylamine	54.4 ± 5.1**	43.7 ± 1.6**	Wako
N-Nitrosodibenzylamine	51.6 ± 7.7**	3.8 ± 2.5**	SIGMA-ALDRICH
N-Nitrosopiperidine	110.5 ± 2.8	97.0 ± 3.8	SIGMA
N-Nitrosopyrrolidine	114.4 ± 2.2	103.4 ± 8.7	SIGMA-ALDRICH
N-Nitrosomorpholine	109.7 ± 4.2	96.7 ± 8.8	SIGMA

N-Nitroso compound at 10 μm was tested and the relative activity in the presence of 40 nM DHT in the Yeast Two-hybrid assay and 0.5 nM DHT in the AR-EcoScreen cell reporter gene assay were calculated as the percentage against DHT without chemicals (%). Values represent the mean ± S.D. ($n = 3$). **$p < 0.01$ compared to DHT without chemicals.

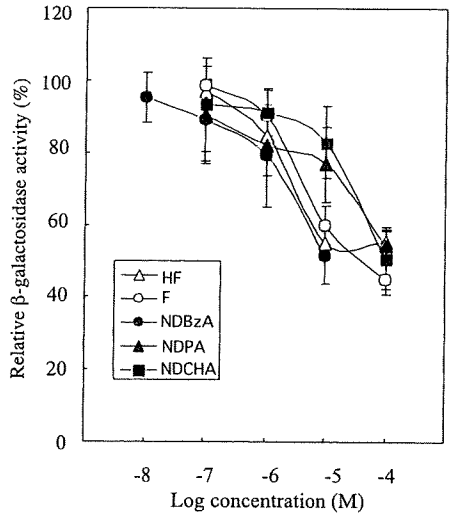


Fig. 2. Dose-Dependent Curves of NOCs in Yeast Two-Hybrid Assay System

Relative β-galactosidase activity of NDBzA, NDPA and NDCHA in the presence of 40 nM DHT was calculated as the percentage against DHT without chemicals (100%). Values represent the mean ± S.D. ($n = 3$).

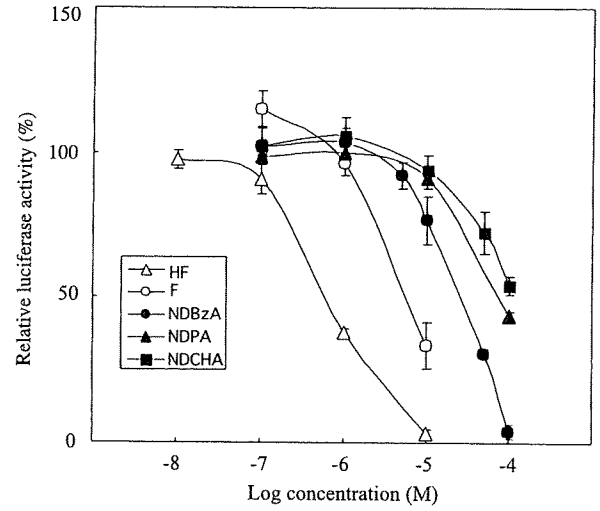


Fig. 3. Dose-Dependent Curves of NOCs in AR-EcoScreen Cell Reporter Gene Assay

Relative luciferase activity of NDBzA, NDPA and NDCHA in the presence of 0.5 nM DHT was calculated as the percentage against DHT without chemicals (100%). Values represent the mean ± S.D. ($n = 3$).

of PSA was about double when the cells were treated with 10 nM DHT for 18 hr, but NDBzA and F decreased the PSA to the same level without DHT. NDPA also decreased the level about 70%, but not NDCHA (Fig. 6).

DISCUSSION

In this study, we assessed the anti-androgenic activity on 56 chemicals using Yeast Two-hybrid assay (AR:SRC-1) and AR-EcoScreen cell reporter gene assay, and found NDPA as a novel anti-androgen. Then we tested on 15 NOCs and found that NDBzA and NDCHA as well as NDPA inhibited the

Table 4. Effects of Compounds on the Inhibition of DHT Activity by the Various Assays

Compounds	Reporter gene assay IC_{50}[a] (μM)	Yeast Two-hybrid assay IC_{50}[a] (μM)	AR binding assay IC_{50}[a] (μM)
F	0.223	4.56	28.6
NDBzA	4.95	2.60	19.9
NDPA	16.9	27.6	183
NDCHA	11.7	54.8	27.4

a) IC_{50} denotes the concentration that chemicals inhibited 50% of DHT without chemicals as described under method.

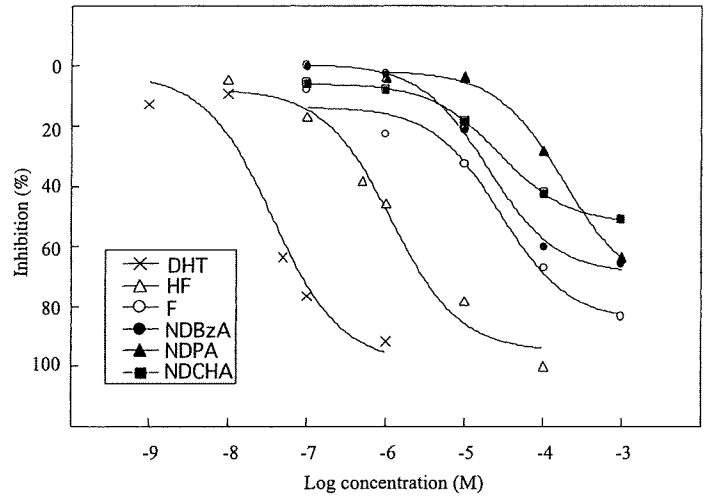


Fig. 4. Competitive Binding of NDBzA, NDPA and NDCHA against AR/AR-Ligand Complex to AR

NDBzA, NDPA and NDCHA competed against Fluormone AL green (fluorescent labeling AR lignads) on binding to human AR. Values represent the mean ± S.D. (n = 3).

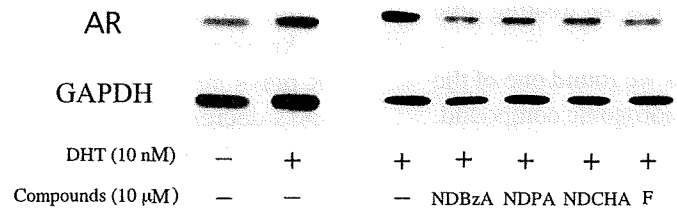


Fig. 5. Effect of NDBzA, NDPA and NDCHA on the Protein Level of AR

LNCaP cells were treated with NDBzA, NDPA and NDCHA in the presence of 10 nM DHT for 10 hr. The level of AR was detected by Western blot analysis.

androgenic activity of DHT.

To estimate the anti-androgenic activity in the Yeast Two-hybrid assay required simpler technique and shorter time, however the sensitivity was lower than the reporter gene assay. Therefore we tested all of the 56 chemicals for anti-androgenic activity by the two *in vitro* assays. Then 9 chemicals found as positive compounds by both. On the other hand, 3 chemicals were positive only by Yeast Two-hybrid assay. The different judgment may be due to difference of used cofactors. Androgen directly interacts with AR, and the complex stimulates transactivation of target genes through interaction with cofactors such as SRC-1, transcription intermediary factor (TIF2), and amplified in breast cancer (AIB1).[23] The AR-EcoScreen cells contain all cofactors, but yeast cells do SRC-1 alone. Another cause may be difference in cell membrane permeability between two

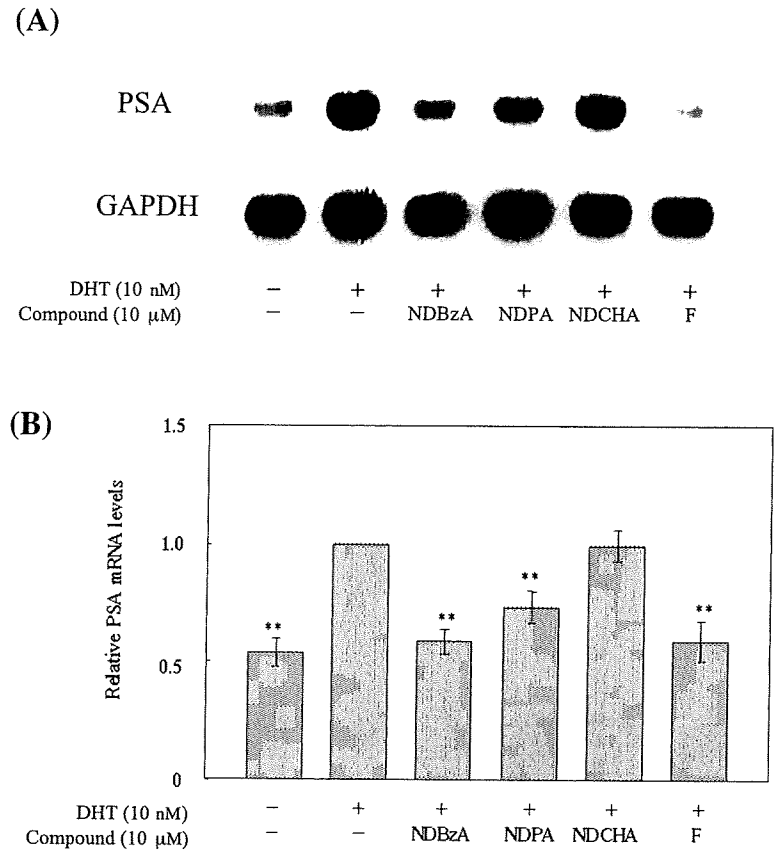


Fig. 6. Effect of NDBzA, NDPA and NDCHA on the Expression of AR Target Gene, PSA

(A) The PSA mRNA levels were determined by Northern blot analysis. LNCaP cells were treated with NOCs in the presence of 10 nM DHT for 18 hr. Total RNA fractions (12 μg each) were subjected to Northern blots. (B) The value of each PSA mRNA level was rectified with the GAPDH. Relative PSA mRNA levels were compared to 10 nM DHT without chemicals (relative PSA mRNA level = 1). Values represent the mean ± S.D. ($n = 3$). $^{**}p < 0.01$ compared to 10 nM DHT without chemicals.

cells and in assay condition such as the treating time of compounds.

Among positive compounds we found one of the NOCs, NDPA, as a novel anti-androgenic compound. Therefore we measured anti-androgenic activity of 15 NOCs to determine whether or not *N*-nitroso group correlated with anti-androgenic activity. As a result NDBzA and NDCHA were positive as well as NDPA, but the others were not. These positive compounds had ring structure other than *N*-nitroso groups. Anti-androgenic activity of NDBzA was estimated the highest, as much as F. NDPA was considered to be higher than NDCHA. These 3 NOCs were competitive in the binding to AR, although binding affinities were low. If the anti-androgenic activity and binding affinity were compared, there is likely to be no correlativity. Because anti-androgenic activity of NDPA was higher than NDCHA, whereas binding affinity of NDPA was less than NDCHA. These results suggest that NDBzA, NDPA and NDCHA antagonized with DHT on the process of competitive binding to AR.

Androgens increase the level of AR protein and AR plays an important role in the nucleus. These NOCs decreased the level of AR, suggesting that NDBzA, NDPA and NDCHA inhibited the androgenic action. The expression of PSA, AR target gene, is regulated by the AR and is thought to function as a growth factor in LNCaP cells.[24–26] Northern blot showed that NDBzA and NDPA inhibited transcriptional level of PSA in LNCaP cells. The same results were obtained by RT-PCR (data not shown). NDBzA inhibited the level of PSA as much as F and NDPA also decreased. These results suggest that 3 NOCs down-regulate the AR target genes mRNA level by antagonizing against DHT in binding process to AR.

This study shows first the anti-androgenic activity of NDBzA, NDPA and NDCHA, that some NOCs may affect endocrine system of humans and wildlife. Thus there are still anti-androgenic compounds that nobody knows. From now on, when we

perform risk assessment of chemicals, it is need to test the androgenic and anti-androgenic effect on endocrine system as well as carcinogenicity and others.

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Dynamic Changes in Dopaminergic Neurotransmission Induced by a Low Concentration of Bisphenol-A in Neurones and Astrocytes

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Key words: bisphenol-A, neurone, astrocyte, dopamine, 17β-oestradiol, drug abuse.

Abstract

One of the most common chemicals that behaves as an endocrine disruptor is the compound 4,4′-isopronylidenediphenol, called bisphenol-A (BPA). We previously reported that prenatal and postnatal exposure to BPA potentiated central dopaminergic neurotransmission, resulting in supersensitivity to psychostimulant-induced pharmacological actions. Many recent findings have supported the idea that astrocytes, which are a subpopulation of glial cells, play a critical role in neuronal transmission in the central nervous system. The present study aimed to investigate the role of neurone–astrocyte communication in the enhancement of dopaminergic neurotransmission induced by BPA. We found that treatment of mouse purified astrocytes and neurone/glia cocultures with BPA *in vitro* caused the activation of astrocytes, as detected by a stellate morphology and an increase in levels of glial fibrillary acidic protein. A low concentration of BPA significantly enhanced the Ca^{2+} responses to dopamine in both neurones and astrocytes. Furthermore, a high concentration of BPA markedly induced the activation of caspase-3, which is a marker of neuronal apoptotic cell death in mouse midbrain neurone/glia cocultures. By contrast, treatment with 17β-oestradiol (E_2) had no such effects. Prenatal and neonatal exposure to BPA led to an enhancement of the dopamine-dependent rewarding effect induced by morphine. These findings provide evidence that BPA alters dopamine responsiveness in neurones and astrocytes and that, at least in part, it may contribute to potentiate the development of psychological dependence on drugs of abuse.

The foetus uses natural hormonal messages that originate in its own hormone system and that of its mother for developmental guidance. Recently, the general public has received alarming reports regarding the reproductive and health hazards of endocrine-disrupting chemicals in the environment. One of the most common endocrine disruptors is the compound 4,4′-isopronylidenediphenol, called bisphenol-A (BPA), which is used in the manufacture of many types of products. These include polycarbonate plastic food storage containers (i.e. baby bottles and water carboys), the lining of food or beverage cans (1, 2), dental composites and sealants and a bioactive bone cement, indicating the potential for human exposure to BPA in daily life.

Our recent studies suggest that exposure to BPA during prenantal and postnatal development has long-lasting effects on central dopaminergic systems linked with behavioural rewarding effects, as well as drug addiction and the reward induced by drugs of abuse (3, 4). The dopamine projection most often linked with a behavioural-rewarding effect is the mesolimbic dopamine system, which originates from the ventral tegmental area (VTA) and terminates at the nucleus accumbens.

We previously demonstrated that prenatal and neonatal exposure to BPA markedly enhanced the rewarding effects induced by drugs of abuse, such as methamphetamine (5) and morphine (6). We also demonstrated that, in adult mice, prenatal and neonatal exposure to BPA enhanced function mediated by central D_1 receptors, which play a substantial role in the rewarding effect of drugs of abuse (5). These treatments also attenuated dopamine function mediated by the D_3 receptor subtype that contributes to the inhibitory modulation of D_1/D_2 receptor-mediated signalling (7, 8). These findings indicate that exposure to BPA during development alters dopaminergic neurotransmission in the central nervous system (CNS), which results in enhancement of the psychological dependence on drugs of abuse. However, the mechanisms underlying these enduring effects of BPA are unknown.

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Many toxic stimuli activate astrocytes, as determined by morphological changes and by an increase in the levels of glial fibrillary acidic protein (GFAP), which is a marker of astrocytes (9, 10). The activation of astrocytes may control the structural and functional plasticity of synapses in the CNS. However, long-term exposure to drugs of abuse can induce neuronal plasticity (11, 12), and we have shown that treatment of mouse cortical neurone/glia cocultures with drugs of abuse, such as methamphetamine and morphine, caused morphological changes in astrocytes (13). Moreover, treatment with methamphetamine increased the sensitivity of astrocytes to dopamine, which is responsible for the rewarding effects of psychostimulants and opioids (13). Together, these findings indicate that astrocytes may play an important role in the development of dependence on drugs of abuse.

Although BPA may affect dopaminergic signalling in the CNS, little is known about the role of BPA in neurone-astrocyte communication. The present study aimed to clarify the effect of BPA in neurone-glia communication. We used mouse midbrain neurone/glia cocultures and purified astrocytes to determine the effects of BPA in the mesolimbic dopamine system.

Because the sex steroid hormones (oestrogens and androgens) have been shown to exert profound effects on brain differentiation, neural plasticity and central neurotransmission (14, 15) and BPA has an affinity for oestrogen receptors, albeit 1 : 2000 that of 17β-oestradiol (E_2) (1), we also investigated the effect of E_2 on astrocytic and neuronal responses and the rewarding effect induced by morphine.

Materials and methods

The present studies were conducted in accordance with the Guide for Care and Use of Laboratory Animals adopted by the Committee on Care and Use of Laboratory Animals of Hoshi University School of Pharmacy and Pharmaceutical Sciences, which is accredited by the Ministry of Education, Culture, Sports, Science and Technology of Japan.

Tissue processing

Purified midbrain astrocytes

Midbrains were dissected from ICR mice at postnatal 1 day (Tokyo Laboratory Animals Science, Tokyo, Japan), minced, and treated with trypsin (0.025%; Invitrogen, Grand Island, NY, USA) dissolved in phosphate-buffered saline (PBS) solution containing 0.02% L-cysteine monohydrate (Sigma-Aldrich, St Louis, MO, USA), 0.5% glucose (Wako Pure Chemicals, Osaka, Japan) and 0.02% bovine serum albumin (Wako Pure Chemicals). After enzyme treatment at 37 °C for 15 min, the cells were dispersed by gentle trituration, collected and centrifuged (20 min, 1000 *g*). After centrifugation, cells were plated in a flask (75 cm^2 culture flask; Corning Inc., Corning, NY, USA). Seven days after seeding in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 5% precolostrum newborn calf serum (FBS, Invitrogen), 5% heat-inactivated (56 °C, 30 min) horse serum (HS, Invitrogen), 10 U/ml penicillin and 10 μg/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C, the flask was shaken for 12 h at 37 °C to remove nonastrocytic cells. Seven days after seeding, the cells were seeded at a density of 1×10^5 cells/cm^3, and maintained for 7 days in DMEM supplemented with 5% FBS, 5% HS, 10 U/ml penicillin and 10^{-5} g/ml streptomycin in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

Midbrain neurone/glia cocultures

Midbrains were obtained from newborn ICR mice at postnatal 1 day, minced, and treated with papain (9 U/ml; Worthington Biochemical, Lakewood, NJ, USA) dissolved in PBS solution containing 0.02% L-cysteine monohydrate, 0.5% glucose and 0.02% bovine serum albumin. After enzyme treatment at 37 °C for 15 min, the cells were dispersed by gentle trituration, collected and centrifuged (10 min, 1000 *g*). The cells were then seeded at a density of 2×10^6 cells/cm^3. The cells were maintained for 7 days in DMEM supplemented with 10% FBS, 10 U/ml penicillin and 10 μg/ml streptomycin. Eight days after seeding, the cells were treated with drugs. In this culture condition, NeuN-positive neurones are surrounded by astrocytes (16).

Drug treatment and immunohistochemistry

Eight days after seeding, *in vitro*, the cells were treated with either normal medium or medium containing bisphenol A (BPA; 10 fM to 1 μM; Wako Pure Chemicals) or 1,3,5[10]-estratriene-3,17β-diol (E_2, 10 fM to 1 μM; Sigma-Aldrich) for 24 h. To explore role for steroid hormone receptors in mediating the effects of BPA (1 pM or 1 μM, 24 h), cells were pretreated with either an oestrogen receptor antagonist 7α,17β-[9[(4,4,5,5,5-pentafluoropentyl)sulfinyl]-nonyl]estra-1,3,5(10)-triene-3.17-diol (ICI182,780; 100 nM, 1 μM or 2 μM; Tocris-Cookson, Ellisville, MO, USA), an oestrogen receptor agonist/antagonist tamoxifen (100 nM, 1 μM or 10 μM; Sigma-Aldrich), a progesterone receptor antagonist mifepristone (100 nM, 1 μM or 10 μM; Sigma-Aldrich) or an androgen receptor antagonist flutamide (100 nM, 1 μM or 10 μM; Sigma-Aldrich) for 24 h. Cells were then treated with normal medium or BPA (1 pM, 1 mM) with or without these steroid hormone receptor ligands for an additional 24 h. Glial cells were then identified by immunofluorescence using mouse anti-glial fibrillary acidic protein antibody (GFAP, dilution 1 : 1000; Chemicon Inc., Temecula, CA, U.S.A), rabbit anti-GFAP (dilution 1 : 1000, Chemicon) or mouse anti-neuronal nuclei (Neu-N) antibody (dilution 1 : 1000, Chemicon) followed by incubation with Alexa 488-conjugated goat anti-mouse IgG (dilution 1 : 4000), Alexa 488-conjugated goat anti-rabbit IgG (dilution 1 : 4000) or Alexa 546-conjugated goat anti-mouse IgG (dilution 1 : 4000). Images were collected using a Radiance 2000 laser-scanning microscope (Radiance 2000; Bio-Rad, Carlsbad, CA, USA).

The intensity of GFAP-like immunoreacivity was measured with a computer-assisted system (NIH Image, Bethesda MD, USA). The upper and lower threshold intensity ranges were adjusted to encompass and match the immunoreactivity to provide an image with immunoreactive material appearing in black pixels, and nonimmunoreactive material as white pixels. The area and intensity of pixels within the threshold value representing immunoreactivity were calculated. We randomly chose 10 areas (80 × 80 pixels) for calculation of GFAP-like immunoreactivity in each image (512 × 512 pixels). The experiments were repeatedly performed by at least three independent culture preparations. The intensity of GFAP-like immunoreactivity was expressed as a percent increase (mean ± SEM) with respect to that in control cells, which were seeded on the same plate.

To evaluate the apoptotic neuronal cell death, mouse midbrain neurone/glia cocultures were treated with normal medium, BPA (1 pM, 1 nM or 1 μM) or E_2 (1 pM, 1 nm or 1 μM) for 24 h. The cells were then identified by immunofluorescence, using rabbit-anticleaved caspase-3 antibody (dilution 1 : 100; Cell Signaling Technology Inc., Beverly, MA, USA), followed by incubation with Alexa 488 conjugated goat anti-rabbit IgG (dilution 1 : 10000). Images were collected using a Radiance 2000 laser-scanning microscope.

Confocal Ca^{2+} imaging

Confocal Ca^{2+} imaging was conducted as previously described (13, 16). Mouse midbrain neurone/glia cocultures or purified astrocytes were incubated for 24 h with normal medium or medium containing BPA (1 pM, 1 nM or 1 μM). Cells were then loaded with 10 μM fluo-3 acetoxymethyl ester (Dojindo Molecular Technologies, Kumamoto, Japan) during a 90-min incubation at room temperature. After a further 20–30 min of de-esterification with the acetoxymethyl ester, the cells which seeded on coverslips were mounted on a microscope equipped with a confocal Ca^{2+} imaging system (Radiance 2000). Fluo-3 was excited with the 488-nm line of an argon-ion laser and the emitted fluorescence was collected at wavelengths > 515 nm, and average baseline fluorescence (F_0) of each cell was calculated. To compensate for the uneven distribution of fluo-3, self-ratios were calculated (ratio: $R_s = F/F_0$). The amplitude was determined by subtracting the average of baseline fluorescence ratio (F_{basal}/F_0) from the maximum of fluorescence ratio after a drug treatment (F_{max}/F_0). Dopamine (1, 10 or 100 μM; Sigma-Aldrich) was perfused via a plastic tube for 30 s at 5 ml/min at room temperature in cultured cortical neurones or astrocytes followed by superfusion of basal salt solution (BSS,

