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8 非ステロイド系医薬品類の生態リスク評価

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Initial Ecological Risk Assessment of Non-steroidal Pharmaceuticals

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要旨：新規の環境微量汚染化学物質として注目される非ステロイド系医薬品類8種類を選定し，ヒメダカ，オオミジンコ，単細胞緑藻類を用いて急性毒性試験に加え慢性毒性試験を実施した．この結果から予測無影響濃度(PNEC)を算出し，環境中からの検出濃度報告(MEC)や国内消費量や未変化体排泄率から求められる予測環境中濃度等(PEC)と比較して生態リスク初期評価をおこなった．その結果，国内での PEC(MEC)/PNEC は全て 0.1 未満となり現時点ではリスク評価作業は必要ないに分類されたため，直ちに水生態系へ影響を与える可能性は低いと考えられる．しかし，acetaminophen 等は海外の MEC との比較によると 0.1 と 1 の間であり，国内での検出データの不十分さや医薬品の生理活性による特異的な慢性影響の可能性があることから，今後より詳細な検討が必要と考えられる．

キーワード：医薬品，ヒメダカ，ミジンコ，生態リスク，慢性毒性
Key Words: Pharmaceuticals, Japanese medaka, daphnia, ecological risk, chronic toxicity

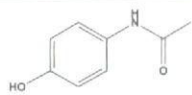
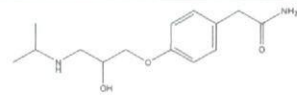
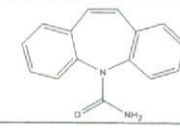
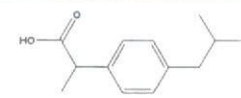
1. はじめに

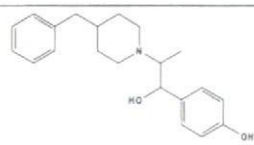
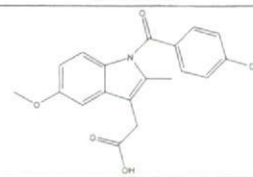
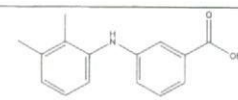
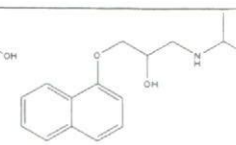
1990 年代後半以降，水環境中に存在して微量で生態系に影響を及ぼす可能性がある物質として，人や家畜が使用する医薬品類が注目されるようになってきた[1-3]．欧州では POSEIDON プロジェクトによって医薬品類の水環境中濃度やその下水・浄水プロセスにおける除去率の大規模な調査が行われた[4]ほか，EMEA によって環境リスクアセスメントも実施されている[5]．米国でも USGS による医薬品類の一斉調査[6]のほか，抗うつ剤が魚体から検出されたという報告[7]による一般市民の関心の高まりを受け，大規模な環境中医薬品類に関するプロジェクト[8]が行われてきた．古典的な水生生物に対する急性・慢性毒性試験についても，欧米各国の様々な研究者によって様々な医薬品類を対象に行われ始めており，昨年にはそのレビューが論文として発行されている[9]．

わが国でも，首都圏を中心に医薬品に関する調査が 2002 年頃から本格化し，下水処理放流水や河川水中から最大 1 μg/L 程度で検出されている[10,11]．また，最近では全国の 27 の一級河川[12]や琵琶湖－淀川水系[13]，近畿圏の下水処理場[14]を対象とした大規模調査の報告もある．一方で水生生物を対象にした毒性試験については，抗生物質クラリスロマイシン[15]や抗菌剤トリクロサン[16]に関する研究例以外は報告が少なく，OECD や環境省が推奨する系統立てた生態リスク評価は実施されていない．岩根ら[17]は国内消費量の多い 87 物質について試行的な生態・環境リスク初期評価スクリーニングを実施し，優先的に評価が必要な医薬品を 8 物質挙げている．しかし，予測無影響濃度(Predicated No Effect Concentration: PNEC)を求める際に実際の毒性試験結果ではなく主に生態学的構造活性相関(ECOlogical Structure Activity Relationship: ECOSAR)を用いた推定値を採用している．また，予測環境

中濃度(Predicted Environmental Concentration: PEC)の算出についても国内消費量等を用いた計算値のみを採用しており，実際の国内からの検出濃度(Measured Environmental Concentration: MEC)については情報不足のために利用されていないなど正確性に問題があった．そこで，われわれは岩根らによる試行的スクリーニング結果[17]のほか，国内の消費量[18]や検出報告[10-14]，海外での医薬品の生態リスク初期評価結果[19-21]などを参考にステロイドホルモン剤や抗生物質を除く非ステロイド系医薬品類8種を選定し，ヒメダカ，オオミジンコ，単細胞緑藻類の3種を用いた急性毒性試験結果からPNECの再計算をおこなってきた[22]．また，下水処理場における除去効率を推定してPECについても計算し，近年の国内でのMECも考慮して，より正確なリスク初期評価を実施した結果を発表[22]している．しかし，低濃度での生理活性が想定される医薬品類については古典的な急性毒性試験だけでは不十分であり，世界的にも非常に報告例が少ない低濃度・長時間曝露を想定した慢性毒性試験の結果を踏まえて生態リスクを評価する必要がある．そこで，本報では環境省が実施している化学物質の生態リスク初期評価[23]を参考にOECDテストガイドラインに準拠してヒメダカとオオミジンコの慢性毒性試験を追加実施し，その結果をもとにPNECを再計算して生態リスク初期評価を実施した結果を報告する．

表1 本研究で使用した医薬品類

項目/医薬品	acetaminophen	atenolol	carbamazepine	ibuprofen
国内年間消費量[a]	740 t	5.4 t	45 t	23 t
未変化体排泄率[b]	0.9-2.7%	90%	2-3%	<1%
化学構造				
PEC/PNEC報告値[c]	0.057	0.7	0.23	0.043

項目/医薬品	ifenprodil	indomethacin	mefenamic acid	propranolol
国内年間消費量[a]	8.6 t	52 t	38 t	1.1 t
未変化体排泄率[b]	20-30%	64%[d] 0.08-0.1%[e]	74%	<1%
化学構造				
PEC/PNEC報告値[c]	0.20	0.28	0.65	0.08

[a]薬事工業動態統計[18]から引用，[b]製薬会社の発行する添付文書から引用，[c]岩根らによる報告[17]から引用，[d]服用剤として使用時，[e]シップ剤として使用時

2. 実験方法

2.1 使用した非ステロイド系医薬品類

使用した非ステロイド系医薬品類は，**表1**に示す acetaminophen（解熱鎮痛剤），atenolol（ベータ

遮断剤), carbamazepine (抗てんかん剤), ibuprofen (非ステロイド系抗炎症剤), ifenprodil tartate (以下 ifenprodil とする：脳循環代謝剤), indomethacin と mefenamic acid (ともに非ステロイド系抗炎症剤), propranolol (ベータ遮断剤) の 8 種とした. 表にそれぞれの年間消費量の推定値と, 未変化体排泄率, 化学構造等を示す. なお, ここで国内年間消費量は厚生労働省医政局編集の「薬事工業動態統計」[18] に示されている値をそれぞれの医薬品含有量等を考慮して足し合わせることによって求めた.

2.2 毒性試験方法

2.2.1 魚類を用いた毒性試験

魚類に対する毒性試験は, 国立環境研究所から分与を受けたヒメダカ(*Oryzias latipes*)を徳島大学内の飼育施設で 2 ヶ月以上馴化・繁殖して用いた. 給餌は 1 日 2 回, ブラインシュリンプ(*Artemia salina*)を与えた. 急性毒性試験は OECD テストガイドライン 203 に準拠して [24] 半止水式で曝露試験をおこない, 96 時間後に半数致死濃度(LC_{50})を測定した. また, 慢性毒性試験は OECD テストガイドライン 210 に準拠して初期発達段階試験を行い [24], 孵化日数, 孵化後 30 日後の全長・体長・湿重量ならびに致死の最大無影響濃度(Maximum NO Effect Concentration: NOEC)を算出した. なお, 水中濃度は島津製作所製の吸光・蛍光検出器付高速液体クロマトグラフィー(HPLC, LC10-AD)を用いて急性試験は試験開始前と終了後, 慢性試験は 1 週間に 1 回以上測定し, 曝露濃度はその測定値の平均値とした.

2.2.2 甲殻類を用いた毒性試験

甲殻類に対する毒性試験には, 国立環境研究所から分与を受けたオオミジンコ (*Daphnia magna*)を徳島大学内の飼育施設で 2 ヶ月以上馴化・繁殖して用いた. 給餌は 1 日 1 回行い, 洗浄済みのクロレラ (川上農場から購入) を与えた. 急性毒性試験である遊泳阻害試験は OECD テストガイドライン 202 に準拠して [24] 止水式で曝露試験をおこない, 48 時間後の遊泳阻害率を調べ, 半数影響濃度(EC_{50})を測定した. 慢性毒性試験は OECD テストガイドライン 211 に準拠して繁殖阻害試験を行い [24], 生後 24 時間以内の幼体を 21 日間飼育し, 遊泳阻害と産仔数の NOEC を算出した. なお, 水中濃度については魚類の試験と同様に HPLC で測定し, その平均値を曝露濃度とした.

2.2.3 藻類生長阻害試験

藻類に対する毒性試験には国立環境研究所から譲与を受けた単細胞緑藻類 (NIES-35, *Psuedokirchneriella subcapitata*)を徳島大学内の飼育施設で 2 ヶ月以上継代・培養して用いた. OECD テストガイドライン 201 に準拠して [24], 照明付インキュベーター内で 100 mL 三角フラスコに入れた AAP 培地内で 96 時間後のブランクに対する成長阻害率を面積法によって算出し, 急性毒性値として EC_{50} を, 慢性毒性値として NOEC を測定した. なお, 曝露濃度は HPLC で測定した初期濃度とした.

2.2.4 ECOSAR を用いた予測

毒性予測値は USEPA から無料でダウンロード可能な EPI Suite の中の ECOSAR v0.99h を用いて, 無機物や界面活性剤など以外の「その他の物質」として対象とした医薬品類 8 種について計算をおこなった. このソフトを用いることによって, 魚類の 96h-LC_{50}, ミジンコ類の 48h-LC_{50}, 緑藻類の 96h-EC_{50} や ChV (NOEC と最小影響濃度 LOEC の幾何平均値) などを算出することができる.

2.3 生態リスク初期評価手法

PNEC (予測無影響濃度) の算出には急性毒性試験の EC_{50} もしくは LC_{50}, さらに慢性試験の NOEC

をアセスメント係数で割ることによって求められる．ここでは，環境省の「化学物質の環境リスク初期評価ガイドライン」[23]をもとにアセスメント係数を設定した（**表2**）．つまり，急性毒性については異なる種族の水生生物3種のデータがあるので，EC_{50}もしくはLC_{50}の最小値をアセスメント係数100で除したもの($PNEC_{acute}$)を求めた．慢性毒性についても同様に3種のデータがあるのでNOECの最小値を10で除したもの($PNEC_{chronic}$)を求めた．なお，ECOSARでの予測値をもとにした$PNEC_{ECOSAR}$についても，同様にアセスメント係数を設定して求めた．PEC（予測環境中濃度）は年間国内消費量，未変化体排泄率（1%未満のものは1%とした），年間国内下水量，活性汚泥による除去率（1%未満のものは1%とした）により下水放流水濃度を予測し(PEC_{domSTP})[22]，さらには希釈率として排出基準と環境基準の考えに基づき10で割ることによって求めた(PEC_{domSW})[22]．また，国内外の検出濃度(MEC)の報告があるものについては下水放流水，河川水に分けて用いた．このように算出したPEC(もしくはMEC)とPNECからPEC(MEC)/PNECを算出した．環境省の「化学物質の環境リスク初期評価ガイドライン」[23]によると，**表3**に示すようにPEC/PNECが0.1を超えた物質についてはさらなる環境影響評価作業が必要とされている．

表2 PNECを算出するためのアセスメント係数[23]

収集可能な情報	推奨されるアセスメント係数
急性毒性試験による（魚類，ミジンコ，藻類のうち）1もしくは2種に対するLC_{50}/EC_{50}	1000
急性毒性試験による3種（通常は魚類，ミジンコ，藻類）に対するLC_{50}/EC_{50}	100
慢性毒性試験による（魚類，ミジンコ，藻類のうち）1もしくは2種に対するNOEC	100
慢性毒性試験による3種（通常は魚類，ミジンコ，藻類）に対するNOEC	10

表3 環境省による化学物質の環境リスク初期評価基準[23]

PEC/PNEC	評価
PEC/PNEC＜0.1	現時点ではそれ以上の作業は必要ないと考えられる
0.1≦PEC/PNEC＜1	情報収集に努める必要があると考えられる
1≦PEC/PNEC	詳細な評価を行う候補と考えられる

表4 魚類，甲殻類，藻類を用いた急性毒性試験の結果[22]

	ヒメダカ 96h-LC_{50}	オオミジンコ 48h-EC_{50}	単細胞緑藻類 96h-EC_{50}
acetaminophen	*800* (258)	17 (41)	2070 (2550)
atenolol	1790 (1460)	*180* (83)	140 (78)
carbamazepine	**20** (102)	**55** (111)	64 (70)
ibuprofen	*89* (32)	31 (39)	*56*< (27)
ifenprodil	4.4 (3.2)	4.1 (2.9)	1.8 (3.4)
indomethacin	*44* (21)	22 (27)	33 (19)
mefenamic acid	*3.6* (1.5)	*10* (2.0)	*17* (1.5)
propranolol	**9.6** (30)	2.7 (2.3)	**0.70** (5.5)

単位はmg/L，カッコ内はECOSAR予測値（ただしヒメダカは魚類96h-LC_{50}，オオミジンコはミジンコ48h-LC_{50}，単細胞緑藻類は藻類96h-EC_{50}），太字は実測値／ECOSAR予測値が0.5以下，*斜体字*は実測値／ECOSAR予測値が2以上

3. 毒性試験結果

3.1 急性毒性試験

表4にヒメダカ，オオミジンコ，単細胞緑藻類を用いた急性毒性試験の結果と，ECOSARによる予測結果を合わせて示す．ECOSARの予測値との差はほぼ1オーダー（0.1倍と10倍の間）以内であった．ifenprodil，mefenamic acid，propranololの3種が他の5種に比べて毒性が強く，特にpropranolol

は藻類とミジンコに対して，mefenamic acid と ifenprodil はメダカに対して 1～10 mg/L のレベルで急性毒性が見られた．残りの 5 種類のうち atenolol については 100 mg/L 以上と急性毒性については比較的弱く，ibuprofen についても水溶解度である 100 mg/L 程度で急性毒性がみられた．acetaminophen は，ミジンコの EC_{50} がメダカや藻類に比べて 2 オーダー低く，オオミジンコや甲殻類に対する特異的な毒性が示唆された．なお，他の研究者での報告がある carbamazepine や propranolol の 3 種に対する急性毒性試験結果[19]，atenolol や ibuprofen の藻類とミジンコに対する急性毒性試験結果[20]，acetaminophen の急性毒性試験結果[21]と比較すると全てその差は 1 オーダー以内でほぼ一致していた．

表5 魚類，甲殻類，藻類を用いた慢性毒性試験の結果

	ヒメダカ 30d-NOEC	オオミジンコ 21d-NOEC	単細胞緑藻類[22] 96h-NOEC
acetaminophen	*180* (41)	**0.14** (28)	*550* (94)
atenolol	270 (NA)	0.83 (NA)	10 (11)
carbamazepine	**6.3** (14)	**2.7** (16d-EC_{50}: 6.4)	6.4 (8.1)
ibuprofen	*15* (5.5)	*9.7* (16d-EC_{50}: 4.3)	**2.0** (7.5)
ifenprodil	**0.046** (0.47)	*0.94* (0.35)	**0.18** (1.0)
indomethacin	*8.5* (3.9)	**0.59** (16d-EC_{50}: 3.6)	**0.84** (6.9)
mefenamic acid	0.35 (0.32)	*1.5* (16d-EC_{50}: 0.43)	*2.5* (1.0)
propranolol	4.2< (NA)	0.066 (NA)	**0.10** (1.4)

単位は mg/L，カッコ内は ECOSAR 予測値（ただしヒメダカは魚類 30d-ChV，オオミジンコはミジンコ 21d-ChV，単細胞緑藻類は藻類 96h-ChV），NA はデータなし，太字は実測値／ECOSAR 予測値が 0.5 以下，*斜体字*は実測値／ECOSAR 予測値が 2 以上

3.2 慢性毒性試験

次に**表5**にヒメダカ，オオミジンコ，単細胞緑藻類を用いた慢性毒性試験の結果を示す．ここで，メダカについては孵化日数よりも 30 日後の体長・致死率等のエンドポイントの方が低濃度で影響がみられたことからその最小値を 30d-NOEC として，ミジンコについては遊泳阻害よりも累積産仔数の方が低濃度で影響がみられたことから産仔数の 21d-NOEC を記した．ifenprodil のメダカや propranolol のミジンコや藻類については 0.1 mg/L 以下の比較的強い毒性を示した．propranolol については魚類生殖毒性が 0.5 μg/L でみられたという報告[25]に比べると，本研究で実測した毒性は弱かった．急性毒性試験と同様に acetaminophen はミジンコに対して毒性が強く，甲殻類に特有の影響を及ぼした可能性がある．atenolol の急性毒性値は全て 100 mg/L 以上と非常に高かったが，ミジンコの慢性値は 1 mg/L を下回るなど一部について急性と慢性毒性に大きな差が見られた．急性毒性値と慢性毒性値の比が acetaminophen，atenolol の甲殻類および ifenprodil の魚類ではアセスメント係数で想定される 10 を大きく上回った．それ以外においても，10 程度もしくはそれ以上となる傾向がみられたため，急性毒性試験によるスクリーニング後，慢性影響を考慮した生態リスク評価を行う必要があると考えられる．

4. 生態リスク初期評価

最後に，これまでの試験結果を総合して生態リスク初期評価を行うため，PEC_{domSTP} と PEC_{domSW} を算出し，国内の下水放流水[10,11,14,26](MEC_{domSTP})，河川水[11-13](MEC_{domSW})，海外の下水放流水[2,27-31]($MEC_{overseaSTP}$)，河川水[2,6,29-31](MEC_{domSW})の最大検出濃度の計 6 種類の値を**表6**に示す．河川水のデータがないものについては，下水放流水の濃度を単純に 10 分の 1 して算出した。**表6**に示すよ

うに PEC や MEC はその方法によって大きく異なり，概して $MEC_{overseaSTP}$ が最も高かった．また，国内の PEC と MEC はほぼ一致しており，予測計算にある程度の精度があると推測される一方で，MEC については最大濃度では下水処理場の処理効率や自然条件が極端な異常値が採用されるという懸念がある．そのため，十分な量のデータを収集し，中央値や 75%もしくは 90%値を収集していく必要性がある．

表6 対象物質の予測環境中濃度と環境中最大検出濃度（μg/L）[22]

	PEC_{domSTP}	PEC_{domSW}	MEC_{domSTP}	MEC_{domSW}	$MEC_{overseaSTP}$	$MEC_{overseaSW}$
acetaminophen	0.071	0.0071	0.025	0.0025	6.2	1.3
atenolol	0.27	0.027	0.00078	0.000078	ND	0.027
carbamazepine	0.10	0.0096	0.45	0.050	2.1	0.36
ibuprofen	0.0034	0.00034	0.18	0.018	1.3	0.29
ifenprodil	0.042	0.0042	0.0021	0.00021	ND	ND
indomethacin	1.4	0.14	0.19	0.019	0.60	0.20
mefenamic acid	0.52	0.052	0.35	0.035	4.5	0.45
propranolol	0.00037	0.000037	0.016	0.0093	0.37	0.10

次に，毒性試験の結果から算出した $PNEC_{exp}$ と ECOSAR 予測値を用いた $PNEC_{ECOSAR}$ の値，そしてそれぞれの値の比を**表7**に示す．表からわかるように，carbamazepine を除く 7 物質については $PNEC_{acute}$ よりも $PNEC_{chronic}$ の方が低く，アセスメント係数が 10 倍異なるにもかかわらず慢性毒性が強い物質が多かった．一方，$PNEC_{exp}/PNEC_{ECOSAR}$ が mefenamic acid 以外の 7 物質について 1 未満になり，ECOSAR の過小評価が指摘されたものの，0.1 を下回るものはなかった．

表7 対象物質の予測無影響濃度（μg/L）

	$PNEC_{acute}$	$PNEC_{chronic}$	$PNEC_{exp}$	$PNEC_{ECOSAR}$	$PNEC_{exp}/PNEC_{ECOSAR}$
acetaminophen	170	14	14	110	0.13
atenolol	1400	83	83	110	0.75
carbamazepine	200	270	200	640	0.31
ibuprofen	310	200	200	270	0.74
ifenprodil	18	4.6	4.6	5.5	0.84
indomethacin	220	59	59	190	0.31
mefenamic acid	36	35	35	15	2.33
propranolol	7.0	6.6	6.6	14	0.47

最後に**表6**と**表7**に示す 6 通りの PEC もしくは MEC と実験によって求めた $PNEC_{exp}$ をもとに生態リスク評価に用いる PEC(MEC)/PNEC 比を求めた．その結果を**図1**に示す．国内についての PEC(MEC)/PNEC 比は岩根らが試行的スクリーニングで算出した値[17]に比べて全ての物質について低く 0.1 を下回った．acetaminophen と mefenamic acid については海外の下水放流水では 0.1 を超える場合もあり，国内での下水放流水や河川水の濃度に関する報告が十分とはいえず，**表3**の判断基準に照らし合わせてみても，今後全国での調査を進めるなど詳細な検討が必要とされる．その他の 6 物質については現時点では水生生物に対して直ちに影響を及ぼすレベルにあるとはいえない．

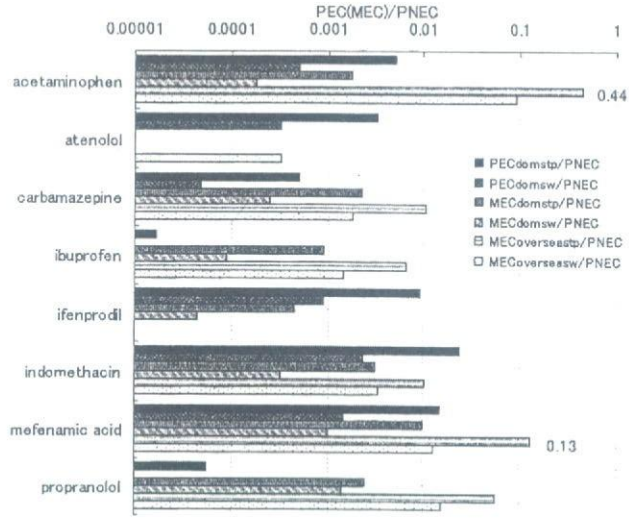


図1 非ステロイド系医薬品類8種の生態リスク初期評価結果

医薬品類の PEC(MEC)/PNEC を求めた海外の研究例は，ドイツの Cleuvers[19]によると atenolol で 0.00077 とほぼ同程度，propranolol で 0.81 と本研究結果の方が 2 から 3 オーダー程度低かった．ドイツとフランスの下水処理場放流水をもとに評価した Ferrari ら[20]によると carbamazepine で最大 2.4，propranolol で最大 104 と 1 をはるかに超えている．欧州での両物質の使用量が多いこと，下水の放流水の最大値をもとに評価していること，carbamazepine はミジンコの繁殖阻害，propranolol は前出の Huggett ら[25]の魚類繁殖阻害が非常に低い濃度で生じていることが原因として考えられる．スイスの下水放流水を用いた ibuprofen の評価[27]では 0.9 と本研究の値とは 3 オーダーの差がある．これは主に ibuprofen の消費量の差のほか，使用している PNEC が 5 μg/L と低いことも原因として考えられる．

生理活性があるようにデザインされている医薬品類は，繁殖毒性や内分泌かく乱，遺伝毒性といった慢性影響が低濃度で起こる可能性もあり，in vitro のスクリーニング試験結果を踏まえて検討する必要がある．また，PEC や MEC については欧米並みの大規模モニタリングの実施はもちろんのこと，下水処理場での挙動・除去効率の正確な推定や，いったん環境中に排出された後の底質，NOM への収着，光分解，生分解についても把握し，総合的な環境リスク管理体制の構築が望まれる．

5. 結論

非ステロイド系医薬品類 8 物質についてヒメダカ，オオミジンコ，単細胞緑藻類の 3 種を用いて急性・慢性毒性試験を実施した結果，国内の PEC(MEC)/PNEC は全て 0.1 以下となり，直ちに水生態系に影響を及ぼすとは考えにくい．しかし，海外の MEC/PNEC が acetaminophen と mefenamic acid で 0.1 を上回ったため，今後費用対効果に応じてより詳細な検討を要する．

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ORIGINAL ARTICLE

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Bisphenol A induces permanent squamous change in mouse prostatic epithelium

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Abstract Bisphenol A (BPA) is a monomer of plastic products widely used in daily life, and has weak estrogenic activity. In this study, male BALB/c mice were treated with BPA and diethylstilbestrol (DES) in adult and fetal periods to investigate whether BPA could affect prostatic epithelial differentiation. Eight-to 9-week-old mice treated for 3 weeks with subcutaneous implants of 0.2–200 mg BPA pellets induced the expression of cytokeratin 10 (CK10) in prostatic basal epithelial cells in a dose-dependent manner. Utilizing organ culture of adult prostate, 1 nM and 1 μM BPA also induced CK10 expression and squamous metaplasia with multilayering of basal epithelial cells, respectively. Fetal exposure to low-dose BPA (20 μg/kg/day) from gestation day (GD) 13 to GD18 induced permanent CK10 expression in basal cells of the adult prostate similar to DES (0.2 μg/kg/day). These results indicate that in mouse, BPA can directly elicit CK10 expression in prostatic epithelium, and that this change can be elicited by doses as low as 20 μg/kg/day. We speculate that low-dose BPA during fetal life may also induce permanent squamous change in human prostate.

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Introduction

Bisphenol A (BPA) is a monomer of polycarbonate plastic products widely used to line metal food and drink cans, as dental sealants and as an additive in many other products. BPA is present as a contaminant in drinking water and has been detected in canned drinks and foods and in the saliva of patients treated with dental sealants. In addition, BPA has been detected in maternal plasma, in the placenta, and in fetal plasma at concentrations of 0.1–10 parts per billion (ppb). Thus, it is likely that the human fetus is exposed to BPA (Schonfelder et al., 2002; Takeuchi et al., 2004; Sugiura-Ogasawara et al., 2005; Welshons et al., 2006). BPA is known to be a weak estrogen both *in vitro* and *in vivo*, and has effects on male and female reproductive organs, mammary gland, brain, behavior, immune function, and pancreatic β-cell function (Markey et al., 2001; Han et al., 2002; Kubo et al., 2003; Tian et al., 2003; Munoz-de-Toro et al., 2005; Alonso-Magdalena et al., 2006; vom Saal and Welshons, 2006). Several reports have showed effects of low-dose BPA, below 50 μg/kg/day, considered to be safe for humans by the United States Environmental Protection Agency (US EPA) (vom Saal and Welshons, 2006; Welshons et al., 2006). Below this dose, BPA had some effects *in vivo* on dairy sperm production, mammary histoarchitecture, seminal vesicle (SV) weight, epididymis weight, age of vaginal opening, and first estrus (vom Saal et al., 1998; Markey et al., 2001; Honma et al., 2002; Chitra et al., 2003; Akingbemi et al., 2004; vom Saal and Welshons, 2006). In the mouse prostate, administration of low-dose BPA (2–50 μg/kg/day) to pregnant female mice produced

a statistically significant increase of prostate weight when assessed in adulthood (Nagel et al., 1997; vom Saal et al., 1998; Gupta, 2000a). However, these low-dose effects on the prostate have not been replicated by others in much larger studies (Ashby et al., 1999; Cagen et al., 1999). Thus, the effect of low-dose BPA on mouse prostate remains controversial. In this regard, the Harvard Center for Risk Analysis reported that there was no consistent affirmative evidence for low-dose effects of BPA on prostatic weight (Gray et al., 2004).

Chronic long-term exposure of high-dose estrogenic substances leads to squamous metaplasia of the prostate, which involves multilayering of the basal epithelial cells and expression of cytokeratin 10 (CK10) (Risbridger et al., 2001b). Estrogen-induced prostatic squamous metaplasia has been described in a large number of species (humans, mice, rats, dogs, sheep, bulls, and goats) (Andersson and Tisell, 1982; Deschamps et al., 1987; Sugimura et al., 1988; Pylkkanen et al., 1991; Weijman et al., 1992; Yonemura et al., 1995). In humans, prostatic squamous metaplasia attributed to high maternal estrone is seen in full-term human male fetuses and newborn male infants. In adulthood, squamous metaplasia is commonly associated with chronic estrogen treatment of patients with benign or malignant prostatic disease (Sugimura et al., 1988; Das et al., 1991; Yonemura et al., 1995; Risbridger et al., 2001a; Shapiro et al., 2005). Induction of squamous metaplasia appears to be elicited via signaling through estrogen receptor-α (ERα) or via growth factor receptor pathways, even though the mechanism is not fully understood (Risbridger et al., 2001a).

In the present study, we investigated effects of BPA on prostatic epithelial cell differentiation in mice using three experimental approaches. First, we treated adult male mice with subcutaneous (s.c.) implants of 0.2–200 mg BPA pellets. Second, explants of adult mouse prostate were treated *in vitro* with BPA in serum-free organ culture to examine direct effects of BPA on the prostate. Finally, the effects of fetal exposure to low-dose BPA were investigated following oral administration of 20 μg/kg/day BPA to pregnant female mice from gestation day (GD) 13–18.

Materials and methods

Animals

Male BALB/c mice were purchased at 7–9 weeks age, and pregnant female BALB/c mice were purchased on the 12th day of gestation from CLEA Japan Inc. (Tokyo, Japan). All animals were housed individually in chip-bedded polyolefin cages in a room with controlled temperature (23 ± 1°C) and humidity (45 ± 65%), on a 12/12 h light/dark cycle. Mice were given phytoestrogen-low diet (NIH-07PLD; Oriental Yeast Co., Tokyo, Japan) and tap water *ad libitum*.

Chemicals

BPA and diethylstilbestrol (DES) with a purity of ≥ 99% were purchased from Nacalai Tesque (Kyoto, Japan) and Wako Pure Chemical Industries (Osaka, Japan), respectively. Cholesterol was purchased from Sigma-Aldrich Co. (St. Louis, MO).

Drug treatment of adult male mice

Male BALB/c mice at 9 weeks age were treated for 3 weeks with s.c. pellets or castrated for 3 weeks as described in Table 1. Dose of BPA was decided as 10-fold series of DES referring to previous study (Risbridger et al., 2001b). All pellets were made in our laboratory using a pellet press (Parr Instrument Company, Moline, IL). Two milligram DES, 0.2 and 2 mg BPA pellets contained cholesterol for adjusting final pellet weights to 20 mg as previously described (Risbridger et al., 2001b). Twenty milligram cholesterol pellets were implanted in control mice. The number of treated mice in each group ranged from seven to nine. Castration and s.c. pellet implantation were performed under anesthesia using 2,2,2-tribromoethanol (Sigma-Aldrich Co).

Table 1 Body weight and reproductive organ weight of adult male mice treated for 3 weeks (mean ± SD)

	Control ($n = 9$)	Castration ($n = 9$)	DES, 2 mg ($n = 8$)	BPA (mg)			
				0.2 ($n = 7$)	2 ($n = 7$)	20 ($n = 7$)	200 ($n = 7$)
Body weight (g)							
Initial	25.50 ± 1.50	24.81 ± 0.73	24.96 ± 0.47	25.22 ± 1.28	24.61 ± 0.92	25.67 ± 0.86	24.10 ± 0.78
Terminal	26.10 ± 0.68	23.42 ± 0.38**	22.95 ± 1.72**	25.77 ± 0.72	25.77 ± 1.15	26.48 ± 0.84	25.49 ± 1.10
Prostate weight (mg)							
AP	42.22 ± 3.93	9.72 ± 5.70**	15.62 ± 9.33**	37.86 ± 9.00	48.22 ± 7.22	41.70 ± 9.05	36.45 ± 5.55
Relative weight	1.52 ± 0.24	0.41 ± 0.24**	0.56 ± 0.45**	1.47 ± 0.35	1.88 ± 0.32	1.58 ± 0.38	1.43 ± 0.23
DLP	24.39 ± 2.70	10.68 ± 4.15**	13.11 ± 9.58*	25.70 ± 8.04	22.61 ± 3.69	21.29 ± 9.07	24.77 ± 7.85
Relative weight	0.86 ± 0.15	0.46 ± 0.18**	0.41 ± 0.36*	1.00 ± 0.34	0.88 ± 0.15	0.82 ± 0.39	0.98 ± 0.32
VP	17.09 ± 2.21	7.02 ± 1.84**	13.24 ± 8.59	14.93 ± 4.28	19.36 ± 3.74	16.23 ± 3.74	19.37 ± 6.30
Relative weight	0.61 ± 0.12	0.29 ± 0.00**	0.46 ± 0.40	0.58 ± 0.18	0.88 ± 0.21	0.62 ± 0.16	0.96 ± 0.40
SV weight (mg)	248.9 ± 44.2	25.5 ± 5.74**	43.7 ± 8.32**	212.0 ± 63.1	221.2 ± 37.1	197.7 ± 7.26*	134.3 ± 25.2**
Relative weight	9.34 ± 1.27	0.30 ± 0.23**	1.72 ± 0.74**	8.18 ± 2.29	8.63 ± 1.65	7.48 ± 0.41*	5.30 ± 1.10**

Relative weight: (organ weight/terminal body weight) × 1000.
*$p < 0.05$: significantly different from the control value by Student's *t*-test.
**$p < 0.01$: significantly different from the control value by Student's *t*-test.
DES, diethylstilbestrol; BPA, Bisphenol A; AP, anterior prostate; DLP, dorsolateral prostate; VP, ventral prostate; SV, seminal vesicle.

Drug treatment of fetal mice

Pregnant female BALB/c mice were treated with 20 μg/kg/day BPA ($n = 3$) or 0.2 μg/kg/day DES ($n = 3$), which were dissolved in a tocopherol-stripped corn oil (MP Biomedicals Inc., Solon, OH) by oral gavage on GD 13–18. As control, the pregnant mice were fed tocophenol-stripped corn oil (2 ml/kg) ($n = 3$). On postnatal day 6, all female pups were discarded, and the number of male pups per litter was adjusted to ≤ 4 pups/female. On postnatal day 42, all male mice were weaned and housed individually until 12 weeks of age. The number of male pups per litter ranged from two to five.

Termination and prostate lobe dissection

All animals were terminated at 12 weeks age by an overdose of isoflurane followed by cervical dislocation. After body weight measurement, prostates were separated into anterior prostate (AP), dorsolateral prostate (DLP), and ventral prostate (VP) lobes under an Olympus SZX7 microscope (Olympus Optical Co., Tokyo, Japan). Wet weights were determined for each prostate lobe and the SV. Tissues were fixed in 10% neutral-buffered formalin (Wako Pure Chemical Industries) for 5 hr for histological and immunohistochemical analyses.

Serum-free organ culture of mouse prostate

Prostates of mice, 8–9 weeks of age, were separated into AP, DLP and VP lobes, and individual prostatic ducts were micro-dissected as previously reported (Sugimura et al., 1986). Individual prostatic ducts were cultured on Millicell CM filters (Millipore Corp., Billerica, MA) floating in medium in six-well culture plates at 37°C in humidified 5% CO_2. The culture medium (phenol-red-free Dulbecco's modified Eagle's medium [DMEM]/Ham's F-12 [1:1]; Invitrogen Corp., Carlsbad, CA) was supplemented with 10 μg/ml insulin (Sigma-Aldrich Co.), 10 μg/ml transferrin (Sigma-Aldrich Co.), 100 U/ml penicillin G, 100 μg/ml streptomycin, and 0.25 μg/ml amphotericin B (Invitrogen Corp.) (Sugimura et al., 1996; Lipschutz et al., 1997; Gupta, 2000a, 2000b). The culture medium containing DES (1 nM) or BPA (1 nM or 1 μM) was poured into each well and exchanged every other day. After 6 days of culture, tissues were harvested, fixed in neutral buffered 10% formalin solution, and processed into paraffin for histological and immunohistochemical analyses.

Immunohistochemistry

Tissues fixed in formalin were dehydrated and embedded in paraffin. Four-micrometer sections were cut using a Leica RM2125 rotary microtome (Leica Microsystems, Wetzlar, Germany). Following deparaffinization, sections were hydrated and incubated for 20 min in 0.3% hydrogen peroxide in methanol. For antigen retrieval, sections were heated in a microwave oven in 0.01 M citrate buffer, pH 6, to 100°C for 10 min. Following a period of cooling, nonspecific binding was blocked using blocking serum (Vectastain *Elite* ABC Kit; Vector Laboratories Inc., Burlingame, CA). Primary antibodies were incubated at 4°C overnight, and then incubated with diluted biotynylated secondary antibody solution at room temperature for 30 min. After washing, sections were incubated with Vectastain *Elite* ABC Reagent (Vector laboratories Inc.) at room temperature for 60 min. Visualization of immunostaining was achieved using 3,3′-diaminobenzidine (DAB). After a rinse in running water, sections were stained with hematoxylin.

To characterize prostatic differentiation, we used anti-cytokeratin 14 (CK14) antibody to identify basal epithelial cells and anti-cytokeratin 10 (CK10) antibody to identify keratinization of the epithelium. Anti-CK14 antibody (LL002; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK) and anti-CK10 antibody (DE-K10; Dako, Copenhagen, Denmark) were used at dilutions of 1:300 and 1:100, respectively. To compare the ERα expression among prostatic lobes, anti-ERα antibody (1D5; Dako) was used at a dilution of 1:20.

RNA extraction and cDNA preparation

The AP, DLP, and VP lobes from 8- to 9-week-old BALB/c mice were dissected in RNA*later* (Ambion, Austin, TX), incubated at 4°C overnight, and stored at −20°C until use. Total RNA extraction from each prostatic lobe was performed by using TRIzol (Invitrogen Corp.) according to the manufacturer's protocol. Total RNA samples were stored at −80°C.

From 1 μg of total RNA, cDNA was reverse transcribed using oligo(dT) (Invitrogen Corp.) and Superscript II RNase H⁻ reverse transcriptase (Invitrogen Corp.) according to manufacturer's protocol. One microgram of total RNA were preincubated with 1 μg of oligo(dT), 4 μl of 2.5 mM dNTP, and distilled water up to 12 μl at 65°C for 5 min. After chilling on ice, 4 μl of 5 × first-strand buffer (final concentration of $MgCl_2$, 3 mM) and 2 μl of 0.1 M DTT were added and incubated at 42°C for 2 min. One microliter of Superscript II RNase H⁻ reverse transcriptase (200 U/μl) was added and incubated at 42°C for 50 min. The reaction was terminated by heating at 70°C for 15 min and stored at −20°C until use.

Real-time polymerase chain reaction (PCR)

Real-time PCR was carried out in the iCycler iQ Detection System (Bio-Rad laboratories, Hercules, CA) using a QuantiTect™ SYBR Green PCR Kit (Quiagen, Valencia, CA) according to the manufacture's protocol. The final 48 μl of PCR master mixture contained 25 μl of 2 × QuantiTect™ SYBR Green PCR master mix (final concentration of $MgCl_2$, 2.5 mM), 1.5 μl each of forward and reverse primers (final concentration of each, 0.3 μM), 20 μl of distilled water, and 2 μl of template cDNA. Amplification reactions were carried out as indicated: initial activation step of 15 min at 95°C to activate Taq DNA polymerase, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s. After PCR, melting curve analysis was performed to verify specificity and identity of the PCR products. Forward and reverse primers for ERα were 5′-GCACAGGATGC TAGCCTTGTCTC-3′ and 5′-AATTGTCACCAGCTTGCAG GT TC-3′, respectively (product size: 106 bp). For glyceraldehyde-3-phosphatase dehydrogenase (GAPDH), the primers were 5′-AAATGGTGAAGGTCGGTGTG-3′ and 5′-TGAAGGGG TCGT TGATGG-3′, respectively (product size: 108 bp) (Takara Bio Inc., Otsu, Japan). To construct standard curves, we prepared 10-fold serial dilutions (five points) of cDNA samples from untreated mouse prostate, and calculated the relative mRNA levels. We used GAPDH as a housekeeping gene for normalization of gene expression. The data were analyzed with the iCycler iQ Optical System Software Ver. 3.0A (Bio-Rad laboratories).

Data analysis

Differences between the two groups were determined using Student's unpaired *t*-test. Findings were considered significant at $p < 0.05$.

Result

Effects on body weight and reproductive organ weight in the adult mouse

Data for mean body and reproductive organ weights ± SD are summarized in Table 1. The body

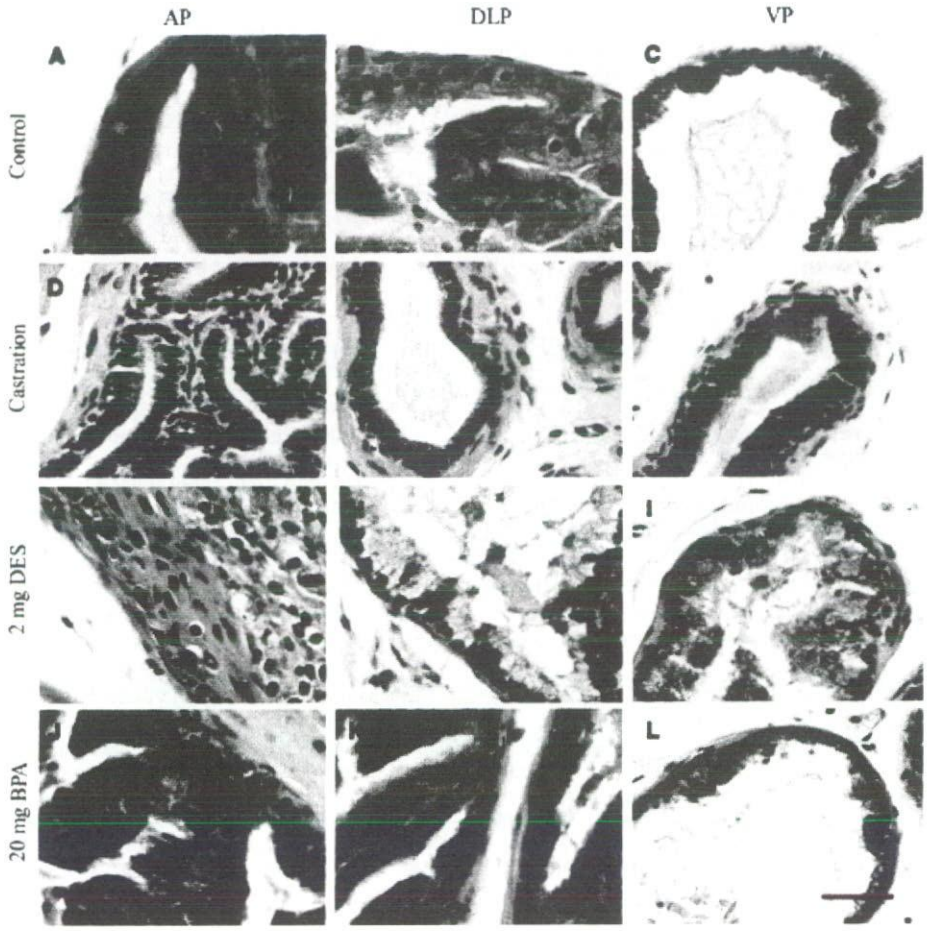

Fig. 1 Effects of diethylstilbestrol (DES) and Bisphenol A (BPA) treatment on adult mouse prostate. All sections are hematoxylin and eosin (H&E) stained. The corresponding sections from anterior prostate (AP), dorsolateral prostate (DLP), and ventral prostate (VP) in control mice are shown in (**A–C**). Effects of castration, 2 mg DES and 20 mg BPA treatment on mouse prostate are shown in (**D–F**), (**G–I**), and (**J–L**), respectively, in order of AP, DLP, and VP. Scale bar, 50 μm, × 400.

weights were significantly decreased in the castration and the DES-treated group, but not in the BPA-treated groups. Prostate weights in the castration group were significantly decreased in all lobes, either absolutely or relative to body weight. AP and DLP weights were significantly decreased in the DES-treated group. There was no effect of treatment with BPA on the prostatic lobe weights. SV wet weight was significantly decreased following castration or treatment with 2 mg DES, or 20 and 200 mg BPA. No effect on the SV weight was seen with 0.2 and 2 mg BPA.

Effect of adult exposure to BPA and DES on mouse prostatic differentiation

In the control group (cholesterol only pellet), prostatic ducts were lined by luminal secretory epithelial cells with basally located nuclei (Figs. 1A–1C). Basal epithelial cells expressing CK14 formed a discontinuous layer underneath the luminal cells along the basement membrane (Fig. 2A). Stromal smooth muscle cells surrounded the prostatic ducts. CK10 was not expressed in any of the lobes of control mice (Fig. 2B).

In castrated mice, the prostatic ducts were shrunken in all lobes (Figs. 1D–1F). The number of CK14-positive basal epithelial cells per unit length of basement membrane appeared to be increased (Fig. 2C). CK10-positive squamous epithelial cells were not detected in any of the prostatic lobes (Fig. 2D).

In the DES-treated group (2 mg pellet), squamous metaplasia was observed in the AP as previously reported (Risbridger et al., 2001b). Squamous metaplasia was characterized by uniform expression of CK14 and CK10 in basal and suprabasal cell layers of a thick stratified squamous epithelium (Figs. 1G,2E,2F) (Risbridger et al., 2001b). In contrast, only patchy areas of CK10 and CK14 positive squamous differentiation were observed in the DLP and VP. Squamous epithelial multilayering was rarely seen in the DLP and VP (Fig. 1H,1I).

Ducts of all prostatic lobes of BPA-treated mice (0.2, 2, 20, and 200 mg pellets) appeared morphologically normal with no apparent morphological alterations as revealed in hematoxylin and eosin (H&E)-stained sec-

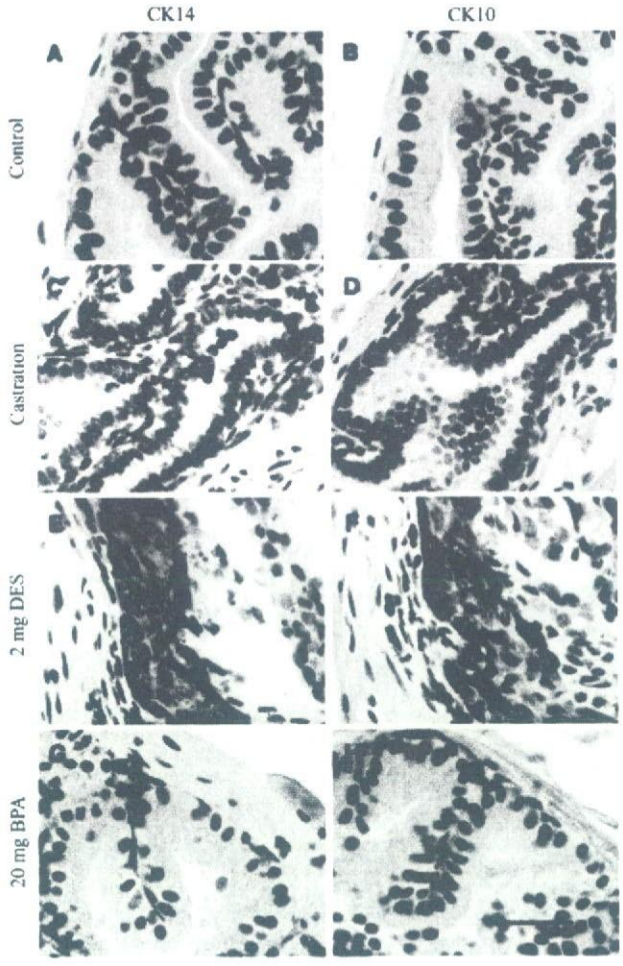

Fig. 2 Immunohistochemical analysis in adult mouse anterior prostate (AP) treated with diethylstilbestrol (DES) and Bisphenol A (BPA). The histopathology was examined with expression of cytokeratin 14 (CK14) (**A**, **C**, **E**, and **G**) and CK10 (**B**, **D**, **F**, and **H**). CK14- and CK10-stained sections from AP in control (A and B), castration (C and D), 2 mg DES (E and F), and 20 mg BPA group (G and H) are shown. Scale bar, 50 μm, × 400.

tions (Figs. 1J–1L). However, at doses delivered from 2 mg BPA pellets and above, CK14-positive basal cells of AP and DLP (but not the VP) co-expressed CK10, more weakly when treated with 2 mg BPA pellets and more strongly when treated with 20 mg BPA pellets (Figs. 2G,2H). In mice treated with 200 mg BPA pellets, basal epithelial cells of all lobes expressed CK10. Therefore, BPA-induced CK10, a classic marker associated with squamous differentiation, was observed in basal cells in a dose-dependent manner. Moreover, each lobe responded differentially to BPA in regard to the induction of CK10; the AP was most sensitive, the VP was the least sensitive and the DLP gave an intermediate response to BPA (Table 2). Immunohistochemical staining in serial sections showed that CK10 was coexpressed

Table 2 Summary of CK10 expression with morphological change in adult exposure

Lobe	Control	Castration	DES, 2 mg	BPA (mg)			
				0.2	2	20	200
AP	–	–	++	–	+	+	+
DLP	–	–	+	–	+	+	+
VP	–	–	+	–	–	–	+

++, CK10 expression with marked multilayering epithelium.
+, CK10 expression without multilayering of epithelium.
DES, diethylstilbestrol; BPA, Bisphenol A; AP, anterior prostate; DLP, dorsolateral prostate; VP, ventral prostate; CK10, Cytokeratin 10.

in CK14-positive basal cells of the BPA-treated group (Fig. 3).

Effects of BPA and DES in serum-free organ culture of mouse prostate

Using a serum-free organ culture system, we examined the direct effect of DES and BPA on the mouse prostate. In control medium lacking estrogenic substances, the epithelial cells of prostatic ducts became cuboidal or low columnar similar to those of prostates of castrated adult male mice (Figs. 4A–4C). CK10-positive squamous cells were not detected in explants from any of the prostatic lobes cultured in control medium (Fig. 5B). However, the number of CK14-positive basal epithelial cells per length of basement membrane appeared to be increased, as was the case for prostates from castrated mice (Figs. 5A,5B).

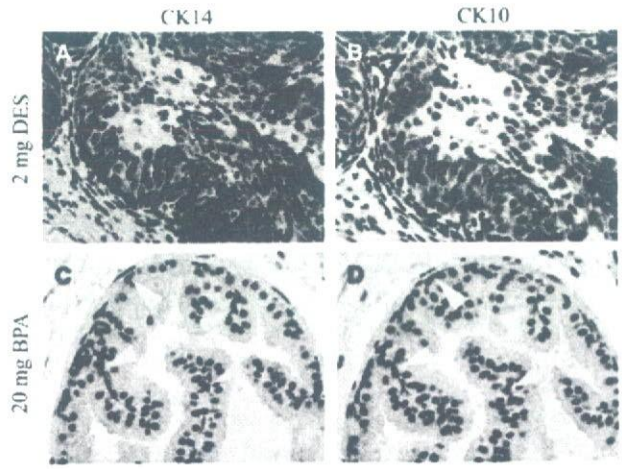

Fig. 3 Coexpression of cytokeratin 10 (CK10) in CK14-positive basal cells of diethylstilbestrol (DES) or Bisphenol A (BPA)-treated mouse anterior prostate (AP). The histopathology in serial sections was examined with expression of CK14 (**A** and **C**) and CK10 (**B** and **D**). CK14- and CK10-stained sections from AP in 2 mg DES (A and B) and 20 mg BPA group (C and D) are shown. Arrowheads indicate the same cells expressing both CK10 and CK14.

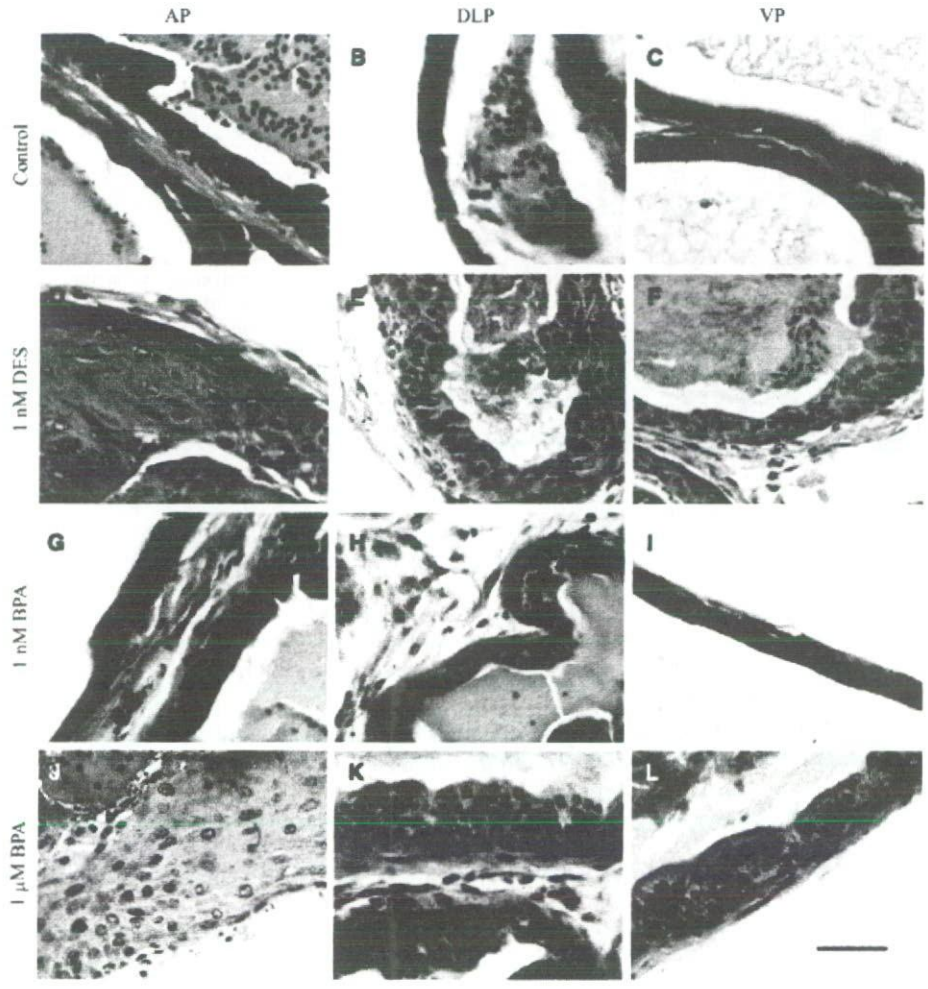

Fig. 4 Effects of diethylstilbestrol (DES) and Bisphenol A (BPA) on adult mouse prostate in serum-free organ culture. All sections are hematoxylin and eosin stained. The corresponding sections from anterior prostate (AP), dorsolateral prostate (DLP), and ventral prostate (VP) in control cultures are shown in (**A–C**). Effects of 1 nM DES, 1 nM and 1 μM BPA on prostatic cultures are shown in (**D–F**), (**G–I**), and (**J–L**), respectively, in order of AP, DLP, and VP. Scale bar, 50 μm, × 400.

Under the influence of DES (1 nM) ducts of the AP became lined with a multilayered squamous epithelium similar to those seen *in vivo* adult male mice treated with s.c. implants of 2 mg DES pellets for 3 weeks (Fig. 4D). In cultures of ducts from the DLP and VP, epithelial stratification and keratinization were weaker than those in the AP (Figs. 4E,4F). In ductal cultures from all lobes, areas of stratification and keratinization were positive for both CK10 and CK14 (Figs. 5C,5D).

Prostatic ducts cultured in the presence of 1 nM BPA (228 parts per trillion [ppt]), which was below that detected in human serum level, exhibited a histology similar to that of control cultures having an atrophic epithelium presumably due to the absence of androgens (Figs. 4G–4I). Most CK14-positive basal epithelial cells appeared to co-express CK10 in ductal cultures derived from all lobes (Figs. 5E,5F). Moreover, when the dose of BPA was increased to 1 μM BPA (288 ppb), squamous changes were observed similar to those seen in 1 nM DES treatment group (Figs. 4J–4L). In culture of AP, squamous metaplasia was more marked as compared with the DLP and VP cultures (Figs. 5G,5H) (Table 3).

Effects of fetal exposure to BPA and DES on prostate

In the control group (corn oil only), a discontinuous layer of basal epithelial cells expressing CK14 was observed on the basement membrane beneath the columnar secretory epithelium similar to prostates of intact mouse. CK10 was not detected and thus prostates from the corn oil-fed group resembled that of normal prostate (Figs. 6A–6C).

In mice treated with DES (0.2 μg/kg/day) and BPA (20 μg/kg/day), prostatic ducts were morphologically indistinguishable from the control group as judged in H&E-stained sections (Figs. 6D,6G). However, in both the DES and BPA treated-group, CK14 positive basal epithelial cells appeared to co-express CK10 in prenatally drug-treated mice aged to 12 weeks (Figs. 6E,6F, 6H,6I).

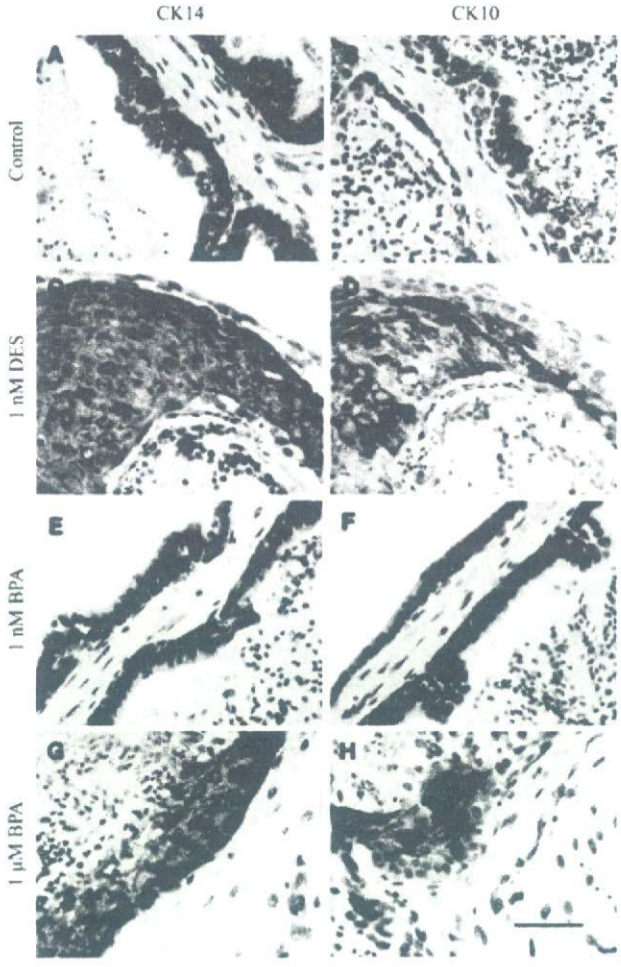

Fig. 5 Immunohistochemical analysis of diethylstilbestrol (DES)- and Bisphenol A (BPA)-treated anterior prostate (AP) in serum-free organ culture. The histopathology was examined with expression of cytokeratin 14 (CK14) (**A**, **C**, **E**, and **G**) and CK10 (**B**, **D**, **F**, and **H**). CK14- and CK10-stained sections from anterior prostate (AP) in control (A and B), 1 nM DES (C and D), 1 nM BPA (E and F), and 1 μM BPA (G and H) are shown. Scale bar, 50 μm, × 400.

Comparison of ERα expression among three prostatic lobes

Based on real-time PCR analysis of AP, DLP, and VP of intact male mice, ERα mRNA expression levels were highest in the AP, lowest in the VP and intermediate in the DLP (Fig. 7A). These findings were corroborated by immunohistochemistry of ERα (Fig. 7B).

Discussion

This study demonstrates that BPA can induce CK10 expression in adult mouse prostatic epithelium in a dose-dependent manner. Organ culture studies indicate that the effects of BPA are direct on the prostate (see also (Jarred et al., 2000)) and that the estrogenic potency of BPA was about 1,000-fold lower than DES as previously reported (vom Saal and Welshons, 2006). Effects of BPA on mouse prostatic epithelium may be manifest as pronounced squamous metaplasia observed morphologically following high-dose exposure or simple induction of CK10 in basal cells underlying the secretory epithelium following low-dose, environmentally relevant exposures to BPA. Lobe-specificity in response to BPA was also observed in mouse prostate, that is, the sensitivity to BPA was highest in the AP and DLP and lower in the VP, which corresponded to parallel differences in the levels of ERα as judged by real-time PCR and immunohistochemistry. Not only adult exposure to high-dose BPA but also fetal exposure to low-dose BPA induced CK10 in prostatic basal epithelial cells. Because the effect of fetal exposure to BPA persisted to 12 weeks postnatal, we consider this to be a permanent effect possibly with long-term deleterious consequences. To our knowledge, this is the first report that squamous changes in mouse prostate can be induced by fetal exposure to low-dose BPA.

Prostatic squamous metaplasia is induced by variety of estrogenic substances in a large number of animal species (Andersson and Tisell, 1982; Deschamps et al., 1987; Sugimura et al., 1988; Pylkkanen et al., 1991; Weijman et al., 1992). Risbridger et al. (2001b) reported that pronounced squamous metaplasia was induced by DES treatment in the AP, and that morphological stratification of prostatic epithelium was associated with extensive expression of CK14 and CK10. One of the earliest changes leading to estrogen-induced squamous metaplasia is the induction of CK10 in basal epithelial cells as previously reported (Risbridger et al., 2001b). Our results demonstrate that while prenatal treatment with low-dose BPA failed to affect prostatic lobe wet weight and failed to elicit any obvious squamous morphological change as judged in H&E sections, CK10 was induced by prenatal BPA and was maintained as an apparent permanent change in mouse prostatic epithelium (at least to 12 weeks postnatal).

Table 3 Summary of CK10 expression with morphological changes in organ culture

Lobe	Control	1 nM DES	1 nM BPA	1 μM BPA
AP	−	+++	+	+++
DLP	−	++	+	++
VP	−	++	+	++

+++, CK10 expression with marked multilayering of epithelium.
++, Focal CK10 expression with marked multilayering of epithelium in partial region.
+, CK10 expression without multilayering of epithelium.
−, No CK10 expression without multilayering of epithelium.
DES, diethylstilbestrol; BPA, Bisphenol A; AP, anterior prostate; DLP, dorsolateral prostate; VP, ventral prostate; CK10, Cytokeratin 10.

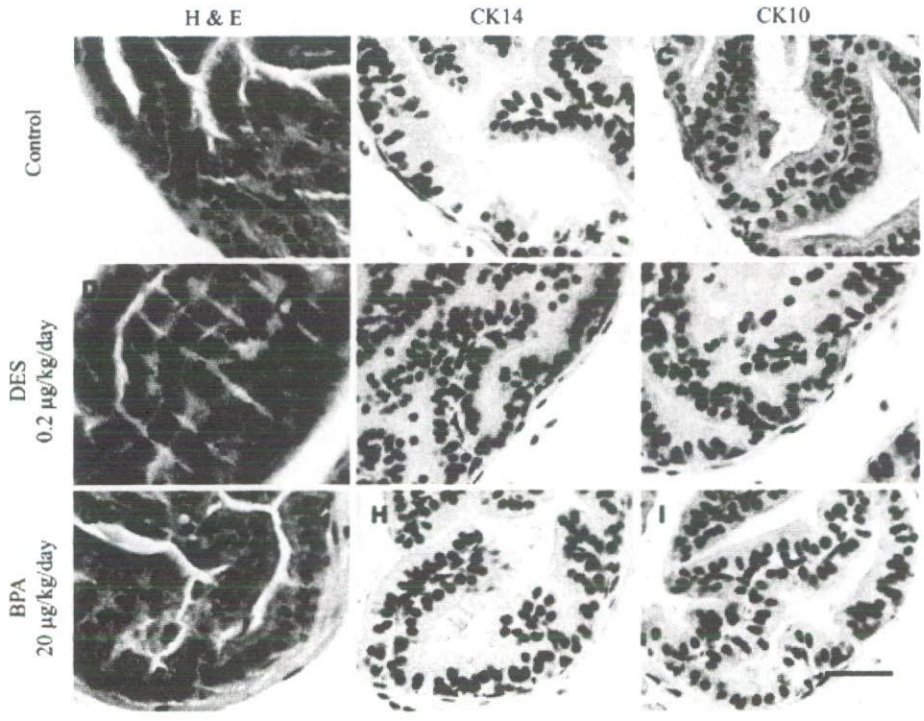

Fig. 6 Effects of fetal exposure to diethylstilbestrol (DES) and Bisphenol A (BPA) on anterior prostate (AP). The histopathology was examined with hematoxylin and eosin (**A**, **D** and **G**) and expression of cytokeratin 14 (CK14) (**B**, **E** and **H**) and CK10 (**C**, **F** and **I**). The corresponding sections from AP in control, DES and BPA group are shown in A–C, D–F, and G–I, respectively. Scale bar, 50 μm, × 400.

Thus, subtle molecular change may not be associated with gross changes in organ weight or gross changes in histodifferentiation.

Substances such as DES and BPA have vastly different estrogenic potencies, which were observed in this study as differential effects of these chemicals on organ wet weight, induction of squamous metaplasia and induction of CK10. It is presumed that effects of DES and BPA especially on expression of squamous metaplasia and CK10 are mediated through ERα. While the mechanism of DES-induced squamous metaplasia is poorly understood, both epithelial and stromal ERα and stromal–epithelial interactions are required (Risbridger et al., 2001a; Cunha et al., 2004). It is likely that squamous change and induction of CK10 elicited by BPA also requires an interaction between epithelium and stroma. The molecular mechanism of estrogen-induced squamous metaplasia may involve paracrine factors of stromal origin that regulate prostatic epithelial differentiation. Growth factors, such as transforming growth factor-β (TGF-β), fibroblast growth factor (FGF), and insulin-like growth factor-1 (IGF-1) are likely paracrine mediators of estrogen action (Chang et al., 1999; Smith et al., 2002; Hong et al., 2004). Further experiments will be required to elucidate the molecular pathways involved in squamous change induced by BPA.

The prostate contains two major epithelial cell types: luminal and basal epithelial cells. Basal epithelial cells are thought to give rise to luminal epithelial cells, and thus prostatic stem cells are thought to reside in the basal cell compartment (De Marzo et al., 1998; Collins et al., 2001; Wang et al., 2001; Hudson, 2004). However, a recent study utilizing p63-knock-out prostate challenges this idea (Kurita et al., 2004). Investigation of the p63-KO model has revealed a critical role of prostatic basal epithelial cells in maintaining ductal integrity and regulating luminal epithelial cell differentiation. For this reason, persistent, possibly irreversible change in gene expression of prostatic basal cells (persistent CK10 expression elicited by fetal low-dose BPA) may have serious long-term consequences. In this regard, alteration in proliferation of prostatic basal epithelial cells has been suggested to be involved in prostate cancer initiation and early progression (Wang et al., 2006). Although possible long-term deleterious consequences of permanent alteration of prostatic basal epithelial cells is unknown, prenatal exposure to estrogenic substances clearly has the potential to elicit precancerous and cancerous lesions (Arai et al., 1978; Santti et al., 1994; Prins, 1997).

Studies have reported effects of low-dose BPA (below 50 μg/kg/day) on prostatic wet weight (vom Saal and Welshons, 2006; Welshons et al., 2006). Fetal exposure to low-dose BPA significantly increased prostate weights of mature mice and rats (Nagel et al., 1997; vom Saal et al., 1998; Gupta, 2000b; Ramos et al., 2001; Chitra et al., 2003). However, other studies utilizing much larger experimental groups have reported no such effect on prostate weights under similar experimental conditions (Ashby et al., 1999; Cagen et al., 1999). In a comprehensive review of the literature through April

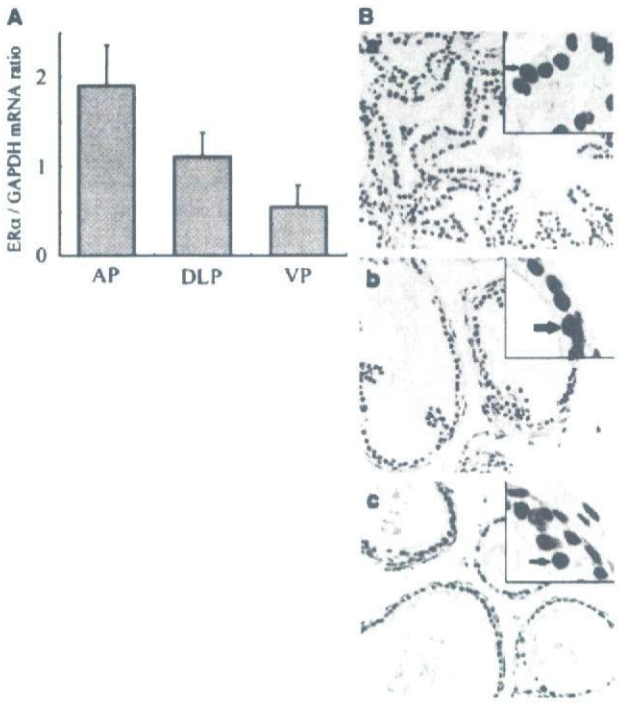

Fig. 7 Comparison of estrogen receptor-α (ERα) expression among prostatic lobes. (**A**) ERα mRNA expression on real-time polymerase chain reaction (PCR) analysis in intact adult mouse prostate. Relative ERα mRNA level relative to glyceraldehyde-3-phosphatase dehydrogenase (GAPDH) level is presented as ERα/GAPDH mRNA ratio (100 × relative ERα/GAPDH mRNA level). ERα/GAPDH mRNA ratios in three lobes are shown. Results represent the average of three experiments performed in triplicate (mean ± SD). (**B**) ERα expression assessed by immunohistochemistry in intact adult mouse prostate. The corresponding sections from anterior prostate (AP), dorsolateral prostate (DLP), and ventral prostate (VP) in intact mice are shown in (a), (b), and (c), respectively.

2002 the Harvard Center for Risk Analysis did not find consistent affirmative evidence for low-dose perinatal exposure to BPA on prostatic wet weight (Gray et al., 2004). The controversy regarding possible effects of low-dose fetal exposure to BPA on prostatic weight is likely to be due to the crude nature of this endpoint and the distinct possibility of interoperator variation in dissecting the mouse prostate. For these reasons, effects of BPA on prostatic wet weight must be viewed with skepticism unless associated with pathological and/or functional changes (Milman et al., 2002). In our study, fetal exposure to low-dose BPA (20 μg/kg/day; GD 13–18) also did not affect prostatic wet weight in adulthood (data not shown). However, our sample size was quite small ($n = 3$ per each group).

Aside from the controversial effects of low-dose BPA on prostate weight, there are reports of low-dose fetal effects of BPA on prostatic development and differentiation (Gupta, 2000a; Ramos et al., 2001; Timms et al., 2005; Ho et al., 2006). Gupta (2000a) reported that low-dose prenatal BPA (50 μg/kg/day; GD 16–18) permanently increased prostatic androgen receptor binding activity in treated offspring. Ramos et al. (2001) reported that low-dose BPA given to pregnant female rats (25 μg/kg/day; GD 8–23) altered the differentiation pattern of the periductal stromal cells of the VP. Timms et al. (2005) reported that low-dose prenatal BPA treatment (10 μg/kg/day; GD 14–18) increased basal epithelial proliferation in the DLP of fetal mice on GD 19. Ho et al. (2006) reported that low-dose BPA treatment of neonatal male rats (0.1 μg/pup or 10 μg/kg by s.c. injection; postnatal day 1, 3, and 5) increased the incidence and susceptibility to neoplastic prostatic lesions at 28 weeks age, and also elicited molecular changes in the prostate during early life before manifestation of histological alterations. We now report that fetal exposure to low-dose BPA induced squamous change (induction of CK10) in prostatic basal epithelial cells, which persisted to 12 weeks age. Endpoints of greater precision encompassing differentiation endpoints in both the stroma and epithelium (CK10) as well as quantified epithelial proliferation and androgen receptor levels now suggest that low-dose fetal exposure to BPA does indeed affect the prostate, that clearly some effects are of a long-term nature, and that some of the consequences of prenatal exposure to low-dose fetal BPA are deleterious. Thus, the risk of BPA exposure should be reassessed (vom Saal and Welshons, 2006).

Difference of response to BPA among three lobes of mouse prostate was the same as that to DES; AP is the most sensitive, DLP is less sensitive, and VP is the least sensitive (Risbridger et al., 2001b). Response to these estrogenic chemicals appears to be mediated via ERα. ERα has been detected in the prostates of several species (Schulze and Barrack, 1987; Prins and Birch, 1997; Yamashita, 2004; Omoto et al., 2005), most often in stromal cells, but also in epithelium (Prins and Birch, 1997; Lau et al., 1998; Risbridger et al., 2001a). Differential response to DES and BPA in the AP, DLP, and VP follows a parallel expression of ERα mRNA levels. Moreover, ERα protein as assessed by immunohistochemistry is highest in the AP, lowest in the VP and intermediate in the DLP. Thus, lobe expression of ERα corresponds reasonably well with biological response to DES and BPA. ERα was rarely detected in the stroma by immunohistochemistry in the adult prostate near ductal tips, but was more commonly seen in the periurethral zone. Failure to detect ERα staining by immunohistochemistry in stromal (or epithelial) cells dose not mean that ERα is absent, as ERα may be present at levels below the sensitivity of the technique. Several studies demonstrated that stromal ERα might play an important role on developmental estrogen effects (Prins et al., 2001; Risbridger et al., 2001a).

There are comparatively little published epidemiologic studies on human health effects of BPA. Two studies describing a relationship between blood levels of

BPA and human gynecological diseases were reported. Takeuchi et al. (2004) reported that serum BPA levels were significantly higher in both nonobese and obese women with polycystic ovary syndrome and obese normal women compared with in nonobese normal women. Sugiura-Ogasawara et al. (2005) reported that human serum BPA levels were higher in patients with a history of recurrent miscarriage relative to control women. There is only one report that BPA could modify both androgen signaling and cellular proliferation in human prostate cancer cells (Wetherill et al., 2002, 2005). The mouse has proven to be a useful experimental model for prostatic biology. The consensus of discussions of mouse prostatic lobar anatomy with human prostatic zonal anatomy (McNeal, 1981; Sugimura et al., 1986) suggests that the mouse DLP is probably homologous with the human peripheral zone where prostate cancer is highest (Price, 1963; Timms et al., 1994). This conclusion is reinforced by gene expression similarities between mouse DLP and human peripheral zone (Berquin et al., 2005). In our mouse model, DLP was intermediate in its sensitivity to BPA relative to the AP and VP. For this reason, the AP and DLP, which express similar secretory proteins (Hayashi et al., 1991), may exhibit progressive deleterious change with time.

In conclusion, induction of CK10 in mouse prostate were elicited in all prostatic lobes by 1 nM BPA *in vitro*, which was below human blood level of BPA, and by 20 μg/kg/day BPA *in vivo*, which was below the safety dose stated by the US-EPA (Schonfelder et al., 2002). We speculate that low-dose fetal exposure to BPA may induce in reversible squamous change in human prostatic basal epithelial cells leading to disease later in life.

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