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H. 知的所有権の出願・登録状況

1. 特許取得
   該当なし
2. 実用新案登録
   該当なし
3. その他
   該当なし

研究成果の刊行に関する一覧表

書 籍

著者氏名	論文タイトル名	書籍全体の編集者名	書籍名	出版社名	出版地	出版年	ページ
舩江良彦	ビスフェノールAによる甲状腺ホルモン撹乱	井口泰泉、井上達編	高次生命系と内分泌撹乱化学物質	シュプリンガー・フェアラーク社	東京	2005年	119-124

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2. ビスフェノールAによる甲状腺ホルモン撹乱

舩江良彦

2. 1. はじめに

内分泌撹乱化学物質による生殖器系への影響に関しては，多くの報告があり，性ステロイドホルモン受容体に作用し，性ステロイド様作用を示すことによって生体へ重大な影響をもたらすと考えられている．一方，ミシガン湖のポリ塩化ビフェニル(PCB)で汚染された魚をたくさん食べた母親や，PCBで汚染された食用油を食べた母親から生まれてきた子供では知能指数の低下，記憶，読解力の低下など脳発達への重大な影響を与えることが報告されている[1,2]．新生児では低甲状腺ホルモン症により脳の各領域の神経細胞の増殖，遊走，神経突起やシナプスの発達およびミエリンの形成に影響を与えることが報告されているので，脳発達への影響に関しては，甲状腺ホルモンの関与が考えられる．しかし，甲状腺ホルモンを撹乱する環境化学物質やそのスクリーニング法などに関してはいまだよくわかっていない．これらの問題を解決するためにも，内分泌撹乱化学物質が甲状腺ホルモン作用を撹乱する機構を明らかにする必要がある．

胎児期および乳児期にビスフェノールA(BPA)に曝露されたマウスでは，行動異常がみられたり，脳内ドパミン量が低下していることをわれわれは報告してきた[3,4]．したがって，BPAが脳の神経細胞に作用すると考えられるので，結合実験によってBPA結合タンパク質がラットの脳P2画分に存在することを確認し，そのタンパク質を精製した．そのBPA結合タンパク質はタンパク質ジスルフィドイソメラーゼ(PDI)として知られているもので，甲状腺ホルモンのリザーバーの機能を有することからBPAが甲状腺ホルモン作用を撹乱する機構が考えられた．その新たな甲状腺ホルモン撹乱機構について述べる．

2. 2. BPA結合タンパク質の検索

ダイオキシンやメチルコラントレンのような多環芳香族炭化水素は強力な発がん性を有することはよく知られている．これらの化合物は細胞内に入り，まずAhRとよばれる多環芳香族炭化水素受容体に結合し，続いて核内に入り，発現調節エレメントのXREに結合し，CYP1A1その他のタンパク質を発現する．われわれはBPAにもAhRのような結合タンパク質が脳内に存在すると考え，結合実験でその存在を検索した．BPA結合タンパク質に対する特異的結合を確認するために，放射性標識リガンドとして^{3}H-BPAを用いて，ラット脳画分とインキュベーションし，B/F分離は遠心分離で行い，放射活性を測定した．ラット脳P2画分に高い特異的結合活性がみられ，BPA結合タンパク質の存在が示唆された．その結果を図2・1に示した．スキャッチャード解析より得られたKd値は23.7 μMと比較的高い値であった．内分泌撹乱化学物質として知られる化合物で，脳発達に影響を与える物質としてPCBなどの報告がみられる．脳発達に影響を与える物質を予測するためにも，また，

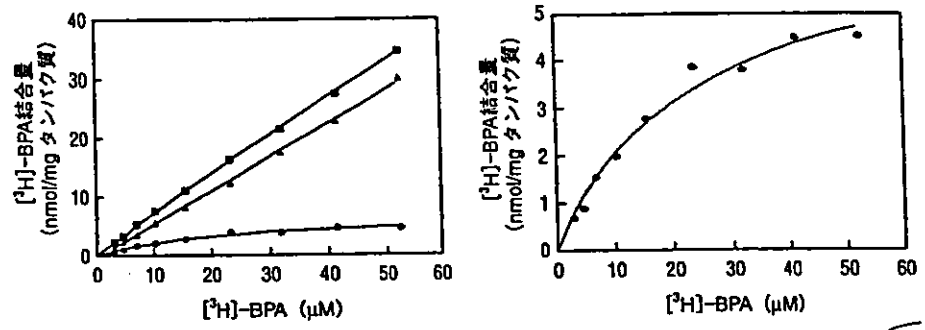

図2・1 ラット脳P2画分に対する[³H]-BPAの特異的結合. ■：全結合, ▲：非特異的結合, ●：特異的結合.【組み込み図, 見えにくいので右に移動しました. 説明をお願いいたします】

その機構を解明するためにも，BPAをはじめそれら化合物に特異的に結合するタンパク質を同定することが重要である．ラット脳P2画分にBPA特異的結合タンパク質の存在が明らかになったので，BPA結合タンパク質の精製を試みた．BPAの特異的結合タンパク質を精製するには，BPAをリガンドとしたアフィニティークロマトグラフィーが最適である．BPAの中央の炭素およびフェノール性OHから6個のメチレン鎖をスペーサーとして導入し，その末端は-NH_2あるいは-COOHとし，セファロース4Bゲルに結合させアフィニティーゲルを作製した．中央のメチルがついた炭素からスペーサーをのばしたゲルが効率よく働いてくれた．ラット脳P2画分を出発材料として，ワットマン社DE-52を用いたイオン交換クロマトグラフィーを行った後，BPAをリガンドとしたアフィニティークロマトグラフィーを行い，BPAで溶出されてくる画分を採取した．SDS-PAGEで単一バンドを示し，分子量は53,000であった．得られたN末端アミノ酸配列はDALEEEDNVLVLKKSNFAEALAAHNYLLVEFYAであった．この配列からBPA結合タンパク質は，ラットのPDIと同定された．

2．3．PDIの機能

PDI(EC5.3.4.1)は，Anfinsenらのグループによって，還元リボヌクレアーゼの再活性化を触媒するタンパク質として初めて見出された[5]．その後，PDIはジスルフィド結合の形成，還元，再構成を行うことが明らかになり，タンパク質の正しいフォールディングのために必須のタンパク質であることが示された[6,7]．PDIは，イソメラーゼ活性を有するだけでなく，シャペロン活性，甲状腺ホルモン脱ヨウ素酵素，甲状腺ホルモン結合タンパク質[8,9]，カルシウム結合タンパク質など，さまざまな機能をもっており"多機能タンパク質"として知られている．

甲状腺ホルモンの細胞内への取り込みは，以前は，受動性の拡散によるものと考えられていた．しかしながら，甲状腺ホルモン結合部位は細胞膜，ミトコンドリア，細胞質，核エンベロープにおいても見出されており，甲状腺ホルモンの取り込みの一部はタンパク質を介した能動輸送であると考えられ，甲状腺ホルモン結合タンパク質の存在が示唆されてきた．山内らは，種々の標的細胞の

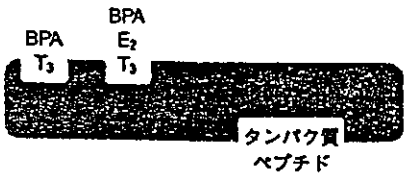

図2・2 PDIの構造．Primm and Gilbert[10]の論文を改変した．E_2：エストラジオール，T_3：3,3',5-L-トリヨードサイロニン，BPA：ビスフェノールA．

膜画分に3,3',5-L-トリヨードサイロニン(T_3)に特異性の高い分子量55,000のタンパク質の存在を見出し，そのタンパク質のcDNAのクローニングを行った．その結果，そのタンパク質はPDIと同一であることを明らかにしてきた[9]．

PDIには，複数のホルモン結合部位が存在することが明らかにされている[10]．その概要を図2・2に示した．異なった2種類のホルモン結合部位と，タンパク質やペプチドが結合する部位の3つの結合部位が存在する．T_3は2つの異なる両ホルモン結合部位に結合するが，エストラジオール(E_2)は片方の部位のみに結合する．PDIに対するこれらのホルモンの結合は，PDIのイソメラーゼ活性を制御している．また細胞内の90%以上のT_3はPDIに結合して存在していると考えられている．われわれの結合実験の結果から，図2・2に示したように，BPAは2つの異なったT_3結合部位に結合することが明らかになった．この結果は，BPAのこれらの結合部位に対する親和性はT_3に比べて低いが，BPAがμM以上になればPDIに結合しているT_3がBPAで置換され，T_3が細胞内へ追い出されると考えられる．すなわち，BPAによって，細胞内T_3レベルが撹乱されることになる．

2. 4. 甲状腺ホルモン撹乱化学物質

PDIに対するBPAおよびその他環境化学物質の結合性について調べるため，ラットPDI遺伝子(*rPDI*)をクローニングし，ヒスチジン融合PDIを大腸菌内で発現し，精製した．このヒスチジン融合PDIに対するT_3の結合が，BPAその他内分泌撹乱性が報告されている環境化学物質によって阻害されるかを競合的結合試験により検討し，IC_{50}値を算出した．B/F分離はポリエチレングリコールを加え遠心分離を行った．競合的結合試験の結果を図2・3に，IC_{50}値は表2・1にまとめた．BPAはT_3のPDIに対する結合を阻害することが明らかになり，その強さはT_3に比べ1/10～1/100程度であった．BPAのエストロゲン受容体に対する親和性はE_2の1/2000ぐらいとの報告がなされているが，それと比べると，BPAのT_3に対する影響はより強く現れると考えられる．BPA以外の環境化学物質では，*p*-ノニルフェノール，ペンタクロロフェノール，テトラブロモBPA，テトラクロロBPAは，T_3のPDIへの結合を阻害することが明らかになった．阻害活性の差こそあるものの，T_3のPDIへの結合阻害活性を有する化合物は，いずれもフェノール基を有しており，構造活性相関が認められた．

PCBが甲状腺ホルモン作用へ影響を及ぼすことは以前より知られていたが，最近になってPCBが甲状腺ホルモン受容体(TR)による転写活性を抑制するといった分子レベルでのメカニズムが明らかにされた[11]．また，BPAもTRに結合し，TR依存性の転写活性を阻害する[12]．また，テトラブロモBPAやテトラクロロBPAもTRに結合し，GH3細胞においてT_3応答性の細胞増殖や成長ホルモンの

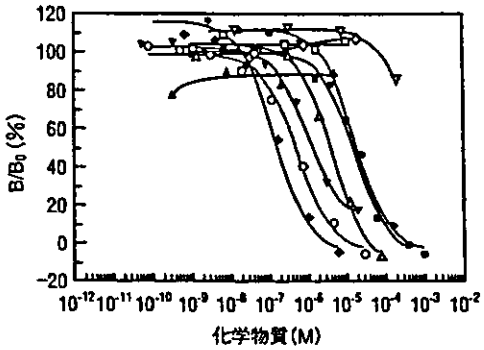

図2・3 3,3',5-L-トリヨードサイロニン競合結合阻害試験. B/B_0：[^{125}I]-3,3',5-L-トリヨードサイロニンの結合率. 被検物質を加えないときの[^{125}I]-3,3',5-L-トリヨードサイロニンの結合量を100%とした. ○：3,3',5-L-トリヨードサイロニン, ●：ビスフェノールA(BPA), △：*p*-ノニルフェノール, ▲：アミトロール, □：トリブチルスズ, ■：ペンタクロロフェノール, ▽：ベンゾフェノン, ▼：テトラブロモBPA, ◇：フタル酸ジエチルヘキシル, ◆：テトラクロロBPA.

表2・1 PDIに対する化学物質の3,3',5-L-トリヨードサイロニン結合阻害

化学物質	IC_{50}
3,3',5-L-トリヨードサイロニン	0.53 μM
ビスフェノールA	17.8 μM
p-ノニルフェノール	4.68 μM
アミトロール	阻害なし
トリブチルスズ	阻害なし
ペンタクロロフェノール	17.4 μM
ベンゾフェノン	阻害なし
テトラブロモBPA	0.96 μM
フタル酸ジエチルヘキシル	阻害なし
テトラクロロBPA	0.15 μM

産生を誘導するとの報告がある[13]. このように, BPA, あるいはBPAのハロゲン化体は, TRを介したgenomicな作用を有することが報告されている. これまで内分泌撹乱化学物質の作用機構は, それらの化学物質が性ステロイドホルモン受容体などの核内受容体に結合することによって, 本来の受容体の活性が撹乱されるgenomicなモデルが提唱されてきた. しかしながら, 甲状腺ホルモンの作用機序はけっしてTRを介したものだけでなく, 細胞膜や細胞質の甲状腺ホルモン結合タンパク質に作用するnongenomicな作用も報告されている[14]. 今回明らかにしたPDIは甲状腺ホルモン結合タンパク質であり, BPAをはじめとする各種環境化学物質がPDIに対するT_3の結合を阻害することによって起こるnongenomicな作用であると考えられる. BPAやテトラブロモBPA, テトラクロロBPAなどは, PDIに結合し, かつTRにも結合する. これらの化学物質は, genomicにもnongenomicにも作用すると考えられる. しかし, *p*-ノニルフェノールやペンタクロロフェノールはTRに対するT_3の結合阻害活性がみられたとの報告はないので, これらの化学物質はPDIを介したnongenomicな作用のみを有すると思われる.

2. 5. ヒトへの影響

ヒトへの影響を考えると，胎児期および授乳期にBPAの曝露を受けると，BPAは血液脳関門から自由に脳内へ取り込まれる．正常な女性の臍帯血や母乳中のBPA濃度にばらつきはあるが，数10 nMという報告がある．甲状腺ホルモンのPDIへの結合定数*Kd*値は数μMであった．缶詰のシーリングや歯科用シーラントに高濃度のBPAが含まれているという報告がみられるので，もし妊娠時に高濃度のBPAを摂取すると，一時的に胎児の脳内BPA濃度が上昇し，PDIに結合している甲状腺ホルモンレベルに影響を与え脳発達に重篤な影響を与えてしまう可能性が考えられる．しかし，胎児期および授乳期を過ぎれば，BPAによる甲状腺ホルモン撹乱による脳発達への影響は少なくなってくると考えられる．

2. 6. おわりに

内分泌撹乱化学物質が甲状腺ホルモン作用を撹乱することによって脳発達へ影響を与えることは明らかである．内分泌撹乱化学物質のターゲットの1つとしてPDIが考えられる．PDIが細胞内で甲状腺ホルモンを蓄えるリザーバーとしての役割を果たしていると考えられているが，内分泌撹乱化学物質がPDIに結合した後，実際どのように甲状腺ホルモンが撹乱されるのかは今後の研究を待たなければならない．また，PDIに対する影響は現在まだBPAなどの一部の環境化学物質についてしか検討されていないが，もっと多くの化学物質について検討されなければならない．内分泌撹乱化学物質の脳発達への影響を検討する場合は，このPDIの関与を考慮する必要があると考えられる．

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Bisphenol A, an environmental endocrine-disrupting chemical, inhibits hypoxic response via degradation of hypoxia-inducible factor 1α (HIF-1α): structural requirement of bisphenol A for degradation of HIF-1α☆

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Abstract

Bisphenol A (BpA), an endocrine-disrupting chemical, is known to be a xenoestrogen and to affect the reproductive functions of animals. Recent reports have documented BpA-induced developmental abnormalities in the neuronal systems of humans and animals, and these effects appear to be non-estrogenic. In this study, we found that BpA inhibited the hypoxic response of human hepatoma cells. The expression of hypoxic response genes such as the erythropoietin (EPO) gene is done via a hypoxia inducible factor 1 (HIF-1)-dependent signaling pathway. To investigate possible structural requirements for this inhibitory effect, several BpA analogs were synthesized and added to this system. The blocking of two phenol groups in BpA did not change the effect, but the inhibition completely disappeared by the removal of two central methyl groups in BpA (the resulting compound is designated BpF). BpA, but not BpF, promoted degradation of the HIF-1α protein, which is a component of HIF-1, followed by inhibition of EPO induction. An immunoprecipitation assay indicated that BpA dissociated heat shock protein 90 (Hsp90) from HIF-1α and destabilized HIF-1α protein. HIF-1α is usually degraded first by ubiquitination and then by the proteasome pathway. Cobalt ion inhibits ubiquitination of HIF-1α and stabilizes it. In the present study, BpA promoted HIF-1α degradation in the presence of cobalt and in the presence of proteasome inhibitor. These results suggest that BpA degraded HIF-1α via a currently unknown pathway, and that this phenomenon required two methyl groups in BpA.

Keywords: Bisphenol A; HIF-α; Hsp90; Erythropoietin

Bisphenol A (BpA) is widely used in industry and dentistry and is one of the most common environmental endocrine disruptors. BpA first became a topic as a xenoestrogen, and there have been many reports on its disrupting effect on estrogenic or sex-related function [1]. Recently, non-estrogenic effects of BpA on the central nervous system have been reported. In the early development of *Xenopus laevis*, BpA has been shown to induce apoptosis in central nervous tissue cells of the brain and spinal cord [2]. In mice, prenatal and neonatal exposure to BpA induces a significant increase in the levels of dopamine D_1 receptor mRNA in the whole brain and enhances central dopamine D_1

☆ *Abbreviations:* BpA, bisphenol A; BpE, bisphenol E; BpF, bisphenol F; BpA-OMe, 2,2-bis(4-methoxyphenyl)propane (dimethyl BpA); HIF-1, hypoxia-inducible factor 1; EPO, erythropoietin; Hsp90, heat shock protein; pVHL, von Hippel–Lindau; Arnt, aryl hydrocarbon nuclear translocator; VEGF, vascular endothelial growth factor; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; SDS, sodium dodecyl sulfate.

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receptor-mediated activity [3]. Despite these intriguing results, however, the molecular mechanism by which BpA induces these phenomena remains unclear.

Hypoxia-inducible factor 1α (HIF-1α) responds to hypoxia by binding to the hypoxia response element (HRE) of target genes, including erythropoietin (EPO), vascular endothelial growth factor (VEGF), and glucose transporter genes [4]. HIF-1α binds HRE motifs as a heterodimeric complex with an aryl hydrocarbon nuclear translocator (Arnt). Both HIF-1α and Arnt are members of a novel subclass of the basic helix–loop–helix (bHLH) family of transcription factors. During normoxia, HIF-1α is hydoxylated on its proline residue by prolyl hydroxylases, resulting in its ubiquitination by von Hippel–Lindau tumor suppressor protein (pVHL) and degradation by proteasome [5,6]. During hypoxia, proline hydroxylation is inhibited and HIF-1α is not ubiquitinated. As a result, HIF-1α protein accumulates and regulates HIF-response genes. HIF-1α, which is overexpressed in common cancers and their metastases, plays essential roles in tumor cell adaptation to hypoxia and angiogenesis [7]. Some chemicals which enhance or inhibit this hypoxic response pathway have been reported [8]. Rotenone and diphenylene iodonium chloride (DPIC) inhibit this pathway. Rotenone is an inhibitor of the mitochondria respiratory chain. Recently, inhibition of the respiratory chain in hypoxia was shown to redistribute oxygen to non-respiratory oxygen-dependent targets, such as prolyl hydroxylase, that induce HIF-1α degradation under hypoxia [9]. In our previous study, we found that DPIC induced the degradation of HIF-1α by binding to NADPH-P450 reductase although the details of this mechanism are not clear [10]. In the current study, we added bisphenol A (BpA), an environmental endocrine-disrupting chemical, to this hypoxic pathway. EPO induction in Hep3B cells under hypoxia was completely inhibited by addition of BpA. EPO, which is a well-known hematopoietic cytokine, is found to be neuroprotective during brain ischemia in adult animal models and also to be required for normal brain development in mouse embryos, suggesting that BpA affects neural development pathway in embryos [11].

In this study, we found that BpA inhibited the induction of hypoxia-responsive proteins such as EPO via the degradation of HIF-1α. We investigated the molecular mechanism of inhibition by BpA on the hypoxic pathway. Furthermore, the essential structure of BpA for this inhibition was identified by the synthesis of BpA analogs.

Materials and methods

Materials. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), penicillin–streptomycin solution, and protease inhibitor cocktail were purchased from Sigma Chemical (St. Louis, MO). Isogen for RNA isolation was purchased from Nippon Gene (Toyama, Japan). RevertAid M-MuLV Reverse Transcriptase was purchased from MBI Fermentas (Vilnius, Lithuania). Ampli Taq Gold was purchased from Applied Biosystems (Foster City, CA). Nitrocellulose membrane, goat anti-rabbit IgG (H+L) horseradish peroxidase conjugate, and 4-chloro-1-naphthol were purchased from Bio-Rad Laboratories (Hercules, CA). The Vectastain ABC kit was purchased from Vector Laboratories (Burlingame, CA). 2,2-Bis(4-hydroxyphenyl)-propane (bisphenol A), 1,1-bis(4-hydroxyphenyl)-ethane (bisphenol E), 4,4′-methylenebisphenol (bisphenol F), and cobalt (II) chloride hexahydrate ($CoCl_2$) were purchased from Wako Pure Chemical Industries (Osaka, Japan). The Anaero Pack (oxygen absorber) was purchased from Mitsubishi Gas Chemicals (Tokyo, Japan). A proteasome inhibitor, MG132, was obtained from Peptide Institute (Osaka, Japan). Antibodies against human Hsp90 and HIF-1α were purchased from BD Biosciences (San Jose, CA) and Novus (Littleton, CO), respectively.

Synthesis of 2,2-bis(4-methoxyphenyl)propane. To a solution of bisphenol A (100 mg, 0.438 mmol) in dimethyl sulfoxide was added sodium hydride (16 mg, 0.657 mmol) under ice cooling. After the reaction mixture was stirred at room temperature for 20 min, iodomethane (40 μl, 0.657 mmol) was added at the same temperature. After the resulting mixture was stirred at room temperature for 10 min, water was added, and then the resulting mixture was extracted with diethyl ether. The organic layers were combined, washed with brine, dried over $MgSO_4$, filtered, and concentrated in vacuo. Purification by silica gel column chromatography (from 2 to 17% ethyl acetate in hexane) gave the 2,2-bis(4-methoxyphenyl)propane (BpA-OMe) (39 mg, 35%) as colorless oils.

^1H NMR ($CDCl_3$, 400 MHz) δ7.14 (AB-q, J = 9.0 Hz, 4H), 6.80 (AB-q, J = 9.0 Hz, 4H), 3.78 (s, 6H), 1.64 (s, 6H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ157.4, 143.1, 127.7, 113.2, 55.2, 41.6, 31.0; IR (NaCl neat, cm^{-1}) 2965, 2836, 1885, 1609, 1510, 1180, 1060, 829; ESI HRMS m/z calcd for $C_{17}H_{20}O_2$ $[M-H]^-$ 255.1385, found 255.1385.

Cell culture. The human hepatoma cell line Hep3B was obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer of Tohoku University (Sendai, Japan). Hep3B cells were maintained in DMEM containing 10% FCS, and 1% penicillin and streptomycin at 37 °C in 5% CO_2.

Treatment. Hep3B cells were cultured in DMEM containing 10% FCS, and the FCS concentration was reduced to 0.1% at 24 h before the treatment with chemicals. For hypoxic treatment, the cells were incubated in 5% O_2, 5% CO_2, and 90% N_2 balanced with a modulator incubator chamber (Napco 7101, Winchester, VA) or were incubated in a sealed 2.5 L box with an Anero Pack for cells. Hep3B cells were incubated for 6 h under hypoxia in the presence of BpA, BpE, BpF, and BpA-OMe (50–200 μM). Hep3B cells were incubated for 6 h in the presence of BpA and BpF (200 μM) after addition of $CoCl_2$ (200 μM).

RNA isolation and reverse transcriptase-PCR. Total RNA was extracted from Hep3B cells with Isogen. A reaction mixture containing 1 μg RNA and 200 U of reverse transcriptase was reacted according to the manufacturer's protocol as follows: incubation for 10 min at 25 °C and 60 min at 42 °C, followed by heating for 10 min at 70 °C to stop the reaction. Polymerase chain reaction (PCR) was performed using a reaction mixture containing 10 pmol of each primer, 1.5 U Ampli Taq, and 100 ng cDNA according to the following protocol: 10 min at 96 °C and then 35 cycles of 30 s at 96 °C, 30 s at 56 °C, and 1 min at 72 °C. Primers for β-actin were 5′-CAAGAGATGGCCACGGCTGCT-3′ (sense) and 5′-TCCTTCTGCATCCTGTCGGCA-3′ (antisense). Primers for EPO were 5′-GCCAGAGGAACTGTCCAGAG-3′ (sense) and 5′-TTCT CCAGGTCATCCTGTCC-3′ (antisense). Primers for HIF-1α were 5′-C TCAACCACAGTGCATTGTA-3′ (sense) and 5′-CAGCACTACTT CGAAGTGGC-3′ (antisense).

Immunoprecipitation and Western blotting. Cells at 80% confluence cultured under normoxia or hypoxia were lysed in 40 mM Tris–HCl buffer (pH 8.0) containing 0.2% Tween 20, 20% glycerol, 10 mM $MgCl_2$, 30 mM KCl, and MG132. Cell lysates were incubated with

HIF-1α or control antibody for 2 h at 4 °C. BpA or BpF was added to the cell lysate together with the antibody. Immunocomplexes were precipitated with protein G. The precipitates were subjected to SDS–polyacrylamide gel electrophoresis with 7.5% polyacrylamide gel. After Western blotting, Hsp90 or HIF-1α proteins were visualized using horseradish peroxidase conjugated to goat anti-rabbit IgG and by 4-chloro-1-naphthol.

Results

Inhibition of EPO induction under hypoxia by BpA

Hep3B cells were cultured under hypoxia. EPO mRNA was strongly induced, and addition of BpA completely suppressed the induction (Fig. 1A). HIF-1α mRNA levels did not change. However, HIF-1α protein levels were reduced by BpA. The inhibitory effect was increased by increase of BpA concentration, and the effects were dose-dependent (Fig. 1B). These results suggest that BpA promoted HIF-1α degradation under hypoxia following the inhibition of EPO induction.

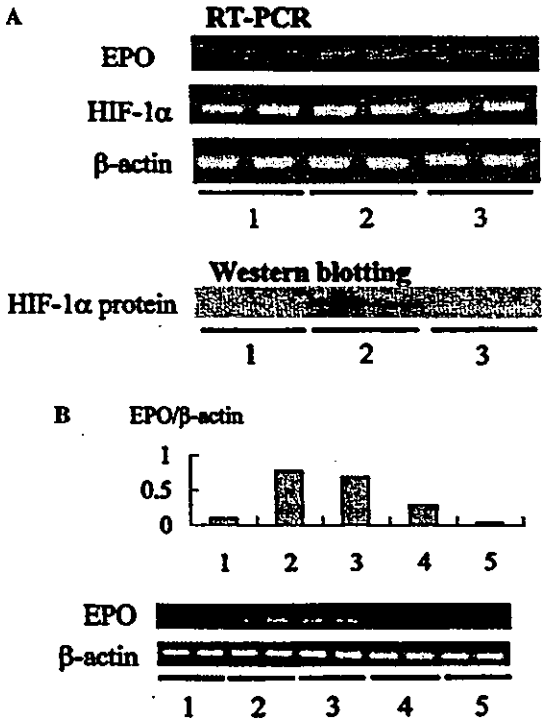

Fig. 1. Inhibition of EPO induction under hypoxia by BpA. (A) Hep3B cells were cultured under hypoxia for 6 h in the absence or presence of BpA (200 μM). Total RNA or protein was isolated from cells in two different culture plates, RT-PCR or immunoblotting was performed. 1, control (normoxia); 2, hypoxia; 3, hypoxia plus BpA. (B) Hep3B cells were cultured for 6 h in the presence of BpA (0, 50, 100, and 200 μM). The graph indicates the relative intensity of the EPO band normalized by β-actin. 1, control (normoxia); 2, hypoxia; and 3, hypoxia plus BpA (50 μM); 4, hypoxia plus BpA (100 μM); and 5, hypoxia plus BpA (200 μM).

Required chemical structure of BpA for inhibition of hypoxic response

The structure of BpA, or 2,2-bis(4-hydroxyphenyl)-propane, consists of two parts, two phenols and propane (Fig. 2A). First, we blocked the hydroxyl groups of phenol by adding methyl groups, and this derivative was added to Hep3B cells under hypoxia (Fig. 2B). The resulting derivative had the same efficiency as the mother compound, BpA. Next, the methyl groups in the propane structure were removed one by one, resulting in bisphenol E (BpE) and bisphenol F (BpF). The inhibitory efficiency was increasingly reduced with the removal of each methyl group (Fig. 2C). These results suggest that the propane structure (two central methyl groups), but not the phenol structure in BpA, was important for the inhibition of EPO induction via the degradation of HIF-1α.

Inhibition of BpA on EPO induction by cobalt

Cobalt ion mimics the hypoxic induction. Cobalt ion inhibits interaction of HIF-1α with pVHL, ubiquitin

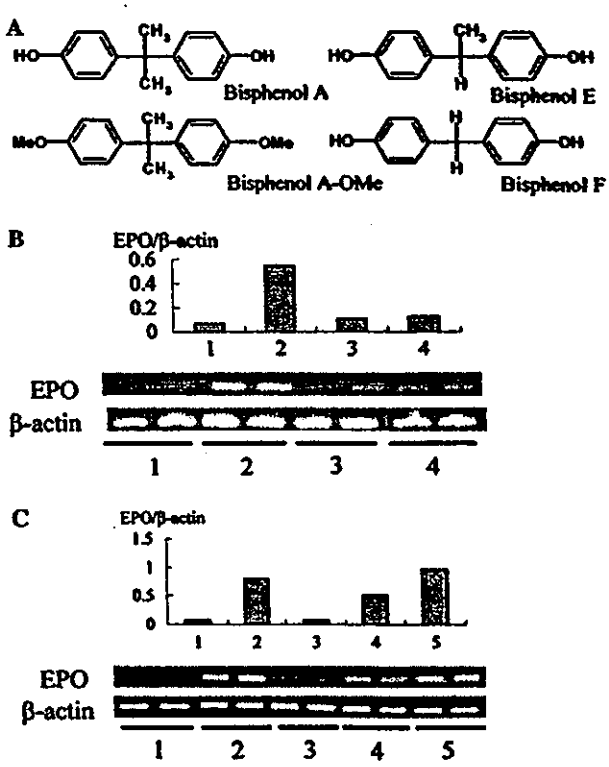

Fig. 2. Relation between BpA chemical structure and inhibition of EPO induction. (A) Chemical structures of BpA analogs. (B) Hep3B cells were cultured for 6 h under hypoxia in the presence of BpA (200 μM) or BpA-OMe (200 μM). 1, control (normoxia); 2, hypoxia; 3, hypoxia plus BpA; 4, hypoxia plus BpA-OMe. (C) Hep3B cells were cultured for 6 h under hypoxia in the presence of BpA, BpE, or BpF (each 200 μM). 1, control (normoxia); 2, hypoxia; 3, hypoxia plus BpA; 4, hypoxia plus BpE; and 5, hypoxia plus BpF.

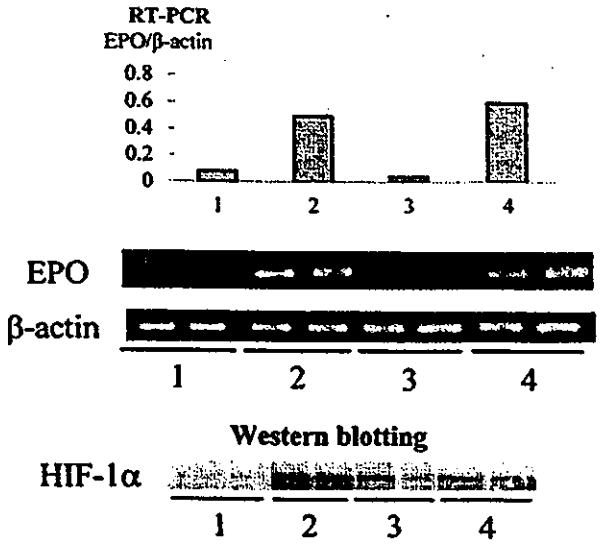

Fig. 3. Inhibition of EPO induction in the presence of cobalt ion by BpA. Hep B cells were cultured in the presence of cobalt ion (200 μM) for 6 h in the absence or presence of BpA (200 μM) or BpF (200 μM). Total RNA or protein was isolated from cells in two different culture plates, and RT-PCR or immunoblotting was performed. The graph indicates the relative intensity of the EPO band on an agarose gel after RT-PCR normalized by β-actin. Immunoblotting using homogenates of Hep3B cells was done with HIF-1α antibody. 1, control (normoxia); 2, treatment with cobalt ion under normoxia; 3, cobalt ion plus BpA; 4, cobalt ion plus BpF.

ligase for HIF-1α [12]. The inhibition produces accumulation of HIF-1α and hypoxic response proteins such as EPO are induced even under normoxia. This process occurs in spite of the prolyl hydroxylation of HIF-1α. BpA was added to Hep3B cells under normoxia with cobalt (Fig. 3). BpA completely inhibited EPO induction by cobalt as in hypoxia. BpF did not inhibit EPO induction, suggesting that the methyl groups in BpA were important for the inhibition. Immunoblotting indicated that BpA but BpF degraded HIF-1α protein in the presence of cobalt ion like in hypoxia. The inhibition pattern by cobalt treatment was the same as that by hypoxia. Hypoxia and cobalt ion stabilize HIF-1α by different mechanisms. Hypoxia inhibits proline hydroxylation in HIF-1α following ubiquitination by pVHL. Cobalt ion inhibits the interaction of HIF-1α with pVHL. BpA inhibited both pathways by inducing degradation of HIF-1α.

Interaction of BpA with Hsp 90 and dissociation of HIF-1α from Hsp90

Mabjeesh et al. [13] found that geldanamycin induces HIF-1α degradation independent of O_2 tension. Recently, it was reported that HIF-1α is associated with the chaperone protein Hsp90 [14]. Geldanamycin is a specific inhibitor for Hsp90. We therefore investigated whether BpA affects the interaction of HIF-1α with Hsp90 (Fig. 4). Hep 3B cells were cultured under

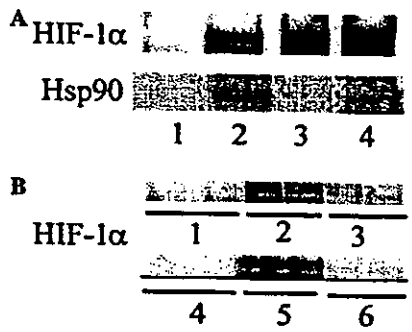

Fig. 4. Effects of BpA and BpF on immmunoprecipitation of HIF-1α and Hsp90 and on degradation of HIF-1α by proteasome. (A) Hep3B cells were cultured for 4 h under normoxia or hypoxia. The cells were homogenized. The homogenates were immunoprecipitated with HIF-1α or control antibodies in the absence or presence of BpA (200 μM) or BpF (200 μM). The precipitate was analyzed by immunoblotting with HIF-1α or Hsp90 antibodies. 1, control (normoxia); 2, hypoxia; 3 hypoxia plus BpA; 4, hypoxia plus BpF. (B) Hep3B cells were cultured under normoxia or hypoxia in the presence of a proteasome inhibitor, MG132 (10 μM). The cells from two different culture plates were homogenized and analyzed by immunoblotting with HIF-1α antibody. 1, control (normoxia); 2, treatment with MG 132 under normoxia; 3, treatment with MG132 and BpA under normoxia; 4 control (normoxia); 5, treatment with MG132 under hypoxia; and 6, treatment with MG132 and BpA under hypoxia.

hypoxia and homogenized. An antibody against HIF-1α or control serum was added to homogenates in the presence or absence of BpA. The resulting antibody complex was precipitated and analyzed by SDS–polyacrylamide gel electrophoresis following immunoblotting with an antibody against Hsp90. As previously reported, HIF-1α and Hsp90 were co-precipitated [14]. However, BpA inhibited the co-precipitation of HIF-1α and Hsp90, indicating that BpA inhibited the function of Hsp90 as in geldanamycin. Furthermore, BpF did not inhibit the co-precipitation, suggesting that the two methyl groups in BpA are required for the interaction of BpA with Hsp90. Moreover, MG132, a proteasome inhibitor, was added to Hep3B cells, and it increased the amount of HIF-1α protein under normoxia and hypoxia (Fig. 3B). The addition of BpA also decreased the amount of HIF-1α protein under both normoxia and hypoxia. These results suggest that the degradation of HIF-1α in Hep3B cells by treatment with BpA occurred via an as-yet-unidentified pathway rather than by the pVHL–proteasome pathway.

Discussion

Bisphenol A (BpA) has physiological effects on reproductive, immunological, and nervous systems in mammals [15–17]. The effects of BpA on the reproducibility or feminization of animals have been extensively studied but the molecular mechanism of the immunological and nervous effects of BpA remains poorly

understood. In this study, we found that BpA inhibited the hypoxic response via the HIF-1 pathway of cells. Genetical deficiency of HIF-1α in mice results in developmental arrest, and the embryos manifest neural tube defects, suggesting that HIF-1α plays an important role in the development of embryos and the formation of neuronal systems [18]. Furthermore, prenatal and neonatal exposure to BpA in mice has been shown to enhance hyperlocomotion, concomitant with an increase of dopamine D_1 receptor mRNA in the whole brain [3]. In vivo, hypoxia decreases dopamine D_1 receptor levels [19]. It is not clear whether the expression of dopamine receptor is regulated by HIF-1, but it can be said that BpA had the opposite effect of hypoxia in this case. EPO is a typical hypoxia-inducible protein and is regulated by HIF-1. EPO is known as a hematopoietic cytokine and it induces erythroid differentiation. It has recently been reported that EPO acts to stimulate neural progenitor cells and to prevent apoptosis in the embryonic brain [11]. Together with these findings, our present results suggest that BpA causes abnormalities of the central neural system in the developmental stage by inhibiting HIF-1 function.

In this study, we found that BpA promoted HIF-1α degradation by dissociation of a chaperon protein, Hsp90, from HIF-1α. The antibiotic geldanamycin has the same effect, inhibiting Hsp90 function followed by HIF-1α degradation. However, the chemical structure of BpA is quite different from that of geldanamycin. Blocking of the phenol groups in BpA by methyl did not affect the inhibition activity or binding activity to Hsp90. However, the two methyl groups in the central position of BpA were important for the binding to Hsp90. Resveratrol, which is similar to BpA and has two phenol structures, also inhibited EPO induction, but it did not interact with Hsp90 (data not shown). Hsp90 recognized the BpA structure selectively. Furthermore, BpA inhibited EPO induction by cobalt, which stabilizes HIF-1α by inhibiting the interaction of HIF-1α with pVHL, a ubiquitin ligase. BpA also enhanced HIF-1α degradation in the presence of cobalt. Isaacs et al. [20] found that geldanamycin enhances HIF-1α degradation in pVHL-deficient cells and suggested that HIF-1α was degraded by a pVHL-independent pathway. BpA also seemed to degrade HIF-1α via a similar pathway. In addition, we found that BpA degraded HIF-1α in the presence of the proteasome inhibitor, MG132, which induced an accumulation of HIF-1α even under normoxia. These results suggest that BpA degraded HIF-1α via an unidentified pathway which is proteasome-independent.

Acknowledgments

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Apigenin suppresses the expression of VEGF, an important factor for angiogenesis, in endothelial cells via degradation of HIF-1α protein

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Abstract Apigenin, a plant-derived flavone, is a potent inhibitor of cell proliferation and angiogenesis, but the mechanisms leading to the pathological anti-angiogenic effects of apigenin are still unclear. In this study, we found that apigenin inhibited the hypoxia-induced expression of vascular endothelial growth factor (VEGF) mRNA in human umbilical artery endothelial cells. Apigenin also suppressed the expression of erythropoietin mRNA, which is a typical hypoxia-inducible gene, via the degradation of hypoxia-inducible factor 1 (HIF-1) α. We investigated the effect of apigenin on the interaction of HIF-1α with heat shock protein 90 (Hsp90), which is reported to be important for the stabilization of HIF-1α, and found that VEGF expression was inhibited via degradation of HIF-1α through interference with the function of Hsp90.

Keywords: Apigenin; Angiogenesis; Vascular endothelial growth factor; Hypoxia; Hypoxia-inducible factor; Heat shock protein 90

1. Introduction

Several polyphenolic compounds are recognized as cancer chemopreventive agents. Flavonoids are especially well known to suppress tumor cell growth via cell-cycle arrest and by the induction of apoptosis in several tumor cell lines [1–5]. Moreover, flavonoids inhibit endothelial cell proliferation and angiogenesis in vitro, the latter is endothelial cell cultures on collagen gels [6,7]. Angiogenesis is essential for the growth, progression, and metastasis of solid tumors [8]. Apigenin, a member of the flavone family that is present at high levels in many vegetables, has been found to inhibit ornithine decarboxylase and chemically induced skin tumorigenesis [9]. However, the mechanism by which apigenin suppresses angiogenesis has not been elucidated.

A prime regulator for angiogenesis is believed to be vascular endothelial growth factor (VEGF) and low oxygen tension dramatically induces the expression of this major angiogenic factor [10]. Transcriptional upregulation of VEGF has been implicated in the induction of genes; the induction is mediated by the specific binding of hypoxia-inducible factor 1 (HIF-1) to the hypoxia response element (HRE) [10–12]. The transcription factor HIF-1 is a heterodimer composed of HIF-1α and aryl hydrocarbon receptor nuclear translocator (ARNT), also known as HIF-1β [13,14]. Although the ARNT protein is readily found in cells, HIF-1α is virtually undetectable in normal conditions. When cells are subjected to hypoxic conditions, the protein levels of the HIF-1α subunit increase rapidly. The existence of HIF-1 suggests that in the presence of oxygen, HIF-1α is regulated by two separate mechanisms: one involving prolyl hydroxylase, which initiates the degradation of HIF-1α, and another involving asparagine hydroxylase, which inactivates the C-terminal transactivation domain of HIF-1α [15]. Under normoxic conditions, the proteasome-dependent degradation of HIF-1α is mediated by prolyl hydroxylation, which permits the binding of the von Hippel–Lindau protein (pVHL), a component of the E3 ubiquitin ligase [16,17]. Under hypoxic conditions, prolyl hydroxylation of HIF-1α is blocked and the transcription factor HIF-1 is stabilized. Detailed study of HIF-1α protein revealed a 200-amino-acid sequence called the oxygen-dependent degradation (ODD) domain [18]. This domain is responsible for the degradation of HIF-1α in the presence of oxygen. pVHL mediates the ubiquitination and degradation of HIF-1α by binding to the ODD domain under normoxic conditions. It has been well documented that cobalt, a transition metal, mimics the effects of hypoxia by stabilizing HIF-1α [19]. However, the biochemical mechanisms underlying this stabilization differ from those underlying the stabilization induced by hypoxia. We have already reported that HIF-1α signal transduction during hypoxia was mediated by NADPH-P450 reductase (NPR) [20]. But NPR has little or no effect on erythropoietin (EPO) mRNA induction by cobalt. A recent study demonstrated that cobalt inhibits HIF–pVHL interaction even after hydroxylation of the proline residue [21], leading to a rapid accumulation of HIF-1α protein.

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Abbreviations: VEGF, vascular endothelial growth factor; HIF-1, hypoxia-inducible factor 1; EPO, erythropoietin; pVHL, von Hippel–Lindau protein; Hsp90, heat shock protein 90; HRE, hypoxia response element; ARNT, aryl hydrocarbon receptor nuclear translocator; ODD, oxygen-dependent degradation; NPR, NADPH-P450 reductase; HUA-EC, human umbilical artery endothelial cells; RT-PCR, reverse-transcription PCR; SDS–PAGE, SDS–polyacrylamide gel electrophoresis; PAS, Per-ARNT-Sim; bHLH, basic helix loop helix

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In this study, we tested the hypothesis that the anti-angiogenic effect of apigenin on tumor cells is caused by a reduction in the expression of VEGF, which is regulated by HIF-1 under hypoxic conditions. Moreover, we investigated a mechanism by which apigenin induces degradation of HIF-1α, independent of the ubiquitination of pVHL.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle's medium (DMEM), EGM-2 medium, fetal calf serum, and *N*-CBZ-Leu-Leu-Norvalinal (proteasome inhibitor) were purchased from Sigma Chemical Co. (St. Louis, MO). Isogen was obtained from Nippon Gene (Toyama, Japan). An RNA PCR Kit (AMV) Ver. 2.1 was purchased from Takara (Shiga, Japan). Anti-human HIF-1α IgG and anti-human ARNT IgG were obtained from Novus (Littleton, CO) and Abcam (Cambridgeshire, UK), respectively. Anti-human heat shock protein 90 (Hsp90) IgG was purchased from BD Biosciences (San Jose, CA). Protein G–Sepharose 4 Faster Flow was supplied by Amersham Biosciences Corp. (Piscataway, NJ). Nitrocellulose membrane and 4-chloro-1-naphthol were purchased from Bio-Rad Laboratories (Hercules, CA). The Vectastain ABC kit (a biotin/avidin system) was obtained from Vector Laboratories (Burlingame, CA).

2.2. Cell culture

The human hepatoma cell line Hep3B was obtained from the Cell Resource Center for Biomedical Research at the Institute of Development, Aging and Cancer, Tohoku University (Miyagi, Japan). Human umbilical artery endothelial cells (HUA-EC) were obtained from Cambrex Bioscience Bio-Whittaker (Walkersville, MD). The Hep3B cells were maintained in DMEM containing 10% fetal calf serum and the HUA-EC in EGM-2 containing 10% fetal calf serum. To stimulate hypoxia, the cells were incubated in 5% O_2, 5% CO_2 and 90% N_2 balanced with a modulator incubator chamber (Napco 7101, Winchester, VA) or in a sealed 2.5-L box with an Anero Pack (oxygen absorber) for cells (Mitsubishi Gas Chemical Company, Inc., Tokyo, Japan).

2.3. Isolation of RNA and reverse-transcription PCR (RT-PCR)

With the use of Isogen, total RNA was extracted from Hep3B cells and from HUA-EC. The total RNA was transcribed into cDNA using the RNA PCR Kit according to the manufacturer's protocol. PCR with 10 pmol of each primer, 1.5 U of Ampli Taq, and 100 ng of cDNA was performed for 10 min at 94 °C and then 35 cycles of 1 min at 94 °C, 1 min at 56 °C and 2.5 min at 72 °C. The PCR primers for EPO [22] were 5′-GCCAGAGGAACTGACCAGAG-3′ (sense) and 5′-TTCTTCAGGTCATCCTATCC-3′ (antisense), while the PCR primers for VEGF [23] were 5′-TTCATGGATGTCTATCAGCG-3′ (sense) and 5′-CATCTCTCCTATGTGCTGGC-3′ (antisense). The oligonucleotide sequences of the reaction products were confirmed by sequencing.

2.4. Western blotting and immunoprecipitation

Cells at 80% confluence were lysed in 20 mM Tris–HCl buffer (pH 8.0) containing 0.1% Tween 20, 10% glycerol, 5 mM $MgCl_2$, 300 mM KCl, and 20 μM *N*-CBZ-Leu-Leu-Norvalinal. Cell lysates were divided into two and incubated with 2.8 μg of anti-HIF-1α IgG at 4 °C. Apigenin (100 μM) was added together with anti-HIF-1α IgG. After 2 h, 60 μL of protein G (10% vol/vol in lysis buffer) was added to each incubation mixture, and the incubation continued for another 2 h. Samples were washed twice by lysis buffer and protein G was removed by centrifugation. Samples were analyzed by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) with 7.5% polyacrylamide gel. Using a previously described method [24], we performed immunoblotting with anti-ARNT IgG, anti-Hsp90 IgG, or antibodies against HIF-1α that were prepared in our laboratory [20]. Each protein was visualized using the ABC kit and 4-chloro-1-naphthol.

3. Results

3.1. Effect of apigenin on VEGF expression under hypoxia

HUA-EC were used to investigate the inhibitory effect of apigenin on VEGF expression (Fig. 1). When HUA-EC were exposed to 5% O_2 for 6 h, VEGF mRNA expression increased markedly. However, VEGF mRNA disappeared completely when apigenin (20 μM) was added to the culture medium. These results show that the flavone apigenin inhibited VEGF mRNA expression under hypoxic conditions.

3.2. Effect of apigenin on EPO expression during hypoxia

To examine whether or not apigenin inhibits the expression of EPO mRNA induced by hypoxia, we used human hepatocyte (Hep3B) cells and analyzed EPO mRNA expression by RT-PCR. The Hep3B cells were exposed to 5% O_2 for 3 h and then the expression of EPO mRNA was observed under hypoxic conditions (Fig. 2A). Apigenin dose-dependently reduced the expression. As HIF-1 was the transcription factor of the EPO gene during hypoxia, we studied the levels of HIF-1α and ARNT in Hep3B cells under hypoxic conditions by Western blotting (Fig. 2B). Although no immunoreactive band for HIF-1α was observed under normoxic conditions in Hep3B cells, HIF-1α was clearly expressed during hypoxia. When apigenin (100 μM) was added to the culture medium, the expression of HIF-1α was reduced. On the other hand, the levels of ARNT, a subunit of HIF-1, were not at all affected by treatment with apigenin.

3.3. Effect of apigenin on EPO expression induced by cobalt

Cobalt is known to inhibit the interaction between HIF-1α and pVHL directly and thereby prevent the degradation of HIF-1α [21]. To examine whether or not apigenin inhibits the cobalt-induced stabilization of HIF-1α, Hep3B cells were treated with apigenin in the presence of cobalt (Fig. 3A). Apigenin inhibited the expression of EPO mRNA induced by cobalt. Moreover, we tested the effect of apigenin on the levels of HIF-1α in Hep3B cells treated with cobalt (Fig. 3B). HIF-1α expression increased on treatment with cobalt, whereas it apparently decreased when apigenin (100 μM) was added to the culture medium. These results indicate that apigenin inhibits the stabilization of HIF-1α in the presence of cobalt. The presence of apigenin did not change the ARNT levels. When apigenin was present, HIF-1α was degraded under hypoxic conditions or in the presence of cobalt ion under normoxic conditions, suggesting that apigenin degraded HIF-1α via a pVHL-independent pathway.

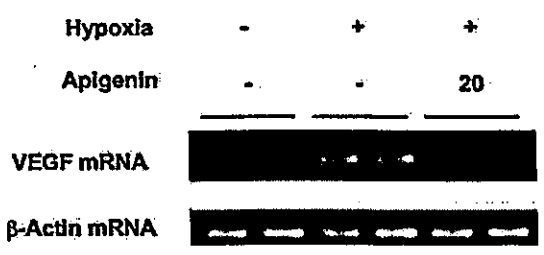

Fig. 1. Expression of VEGF mRNA in HUA-EC. HUA-EC were exposed to 5% O_2, 5% CO_2 and 90% N_2 for 6 h in the presence or absence of 20 μM apigenin. VEGF mRNA expression was analyzed by RT-PCR.

3.4. Hsp90 is required for the stabilization of HIF-1α during hypoxia

Using geldanamycin, a specific inhibitor of Hsp90, Minet et al. [25] demonstrated that Hsp90 was essential for stabilizing HIF-1. In addition, HIF-1α belongs to the Per-ARNT-Sim (PAS)-basic helix loop helix (bHLH) family and interacts with Hsp90 [25,26]. We hypothesized that the degradation of HIF-1α by apigenin correlates with the interaction between HIF-1α and Hsp90. We prepared cell lysates from Hep3B under conditions of hypoxia in the absence of apigenin. Apigenin was added to the lysates together with anti-HIF-1α IgG and then the complex anti-HIF-1α IgG was collected using protein G. The samples were analyzed by SDS–PAGE with 7.5% polyacrylamide gel. The antibody against HIF-1α could pull down Hsp90 protein, indicating that Hsp90 is associated with HIF-1α (Fig. 4). When apigenin was added to the cell lysates together with the antibody, the band of Hsp90 disappeared, indicating that apigenin inhibited the interaction of HIF-1α with Hsp90. Geldanamycin also reduced the levels of EPO mRNA in Hep3B cells under hypoxic conditions (data not shown).

4. Discussion

Dietary factors contribute to about one-third of potentially preventable cancers, and the preventive effects of plant-based diets on tumorigenesis and other chronic diseases have been well documented [27]. Several cancers, including breast cancer, have a lower incidence in Asia than in Western countries. This has been attributed to the Asian dietary regimen, which is typically rich in flavonoid-containing plants. Researchers have identified the isoflavonoid genistein as a potent inhibitor of angiogenesis in vitro [6,7]. A recent study demonstrated that the anti-angiogenic effect of genistein was due to inhibition of HIF-1, an important regulator of VEGF gene homeostasis particularly under low-oxygen conditions [28]. The flavone apigenin has also been proposed as an antitumor agent [29]. However, the molecular mechanisms involved in the anti-angiogenic effects of apigenin are not well understood. This study investigated the anti-angiogenic effects of apigenin on cells under hypoxic conditions. We observed that apigenin suppressed the expression of both VEGF mRNA and EPO mRNA induced by hypoxia in HUA-EC and in Hep3B cells. Apigenin proved more effective against the expression of VEGF mRNA and EPO mRNA than genistein (data not shown). In this study, we found that apigenin caused the degradation of HIF-1α but not of ARNT. Given that genistein has been reported to inhibit HIF-1 DNA-binding activity [28], apigenin and genistein may have different angiogenic mechanisms.

Apigenin inhibited the mRNA expression of EPO induced by cobalt, which mimics hypoxia; it also inhibited the stabilization of HIF-1α induced by cobalt. Because the mechanism underlying the stabilization of HIF-1α during hypoxia is known to differ from that induced by cobalt, we speculate that the degradation of HIF-1α by apigenin is independent of the ubiquitination by pVHL. As geldanamycin reduced the expression of EPO mRNA in Hep3B cells under hypoxic conditions (data not shown), we examined the effect of apigenin on the interaction with HIF-1α of the chaperone protein Hsp90, which associates with HIF-1α to stabilize an activated form of HIF-1 during hypoxia [25,30]. Through immunoprecipitation experiments, we found that apigenin disturbed the binding of Hsp90 to HIF-1α. These results indicate that apigenin diminishes the hypoxia-induced interaction of HIF-1α with Hsp90 to release HIF-1α, which in turn undergoes degradation.

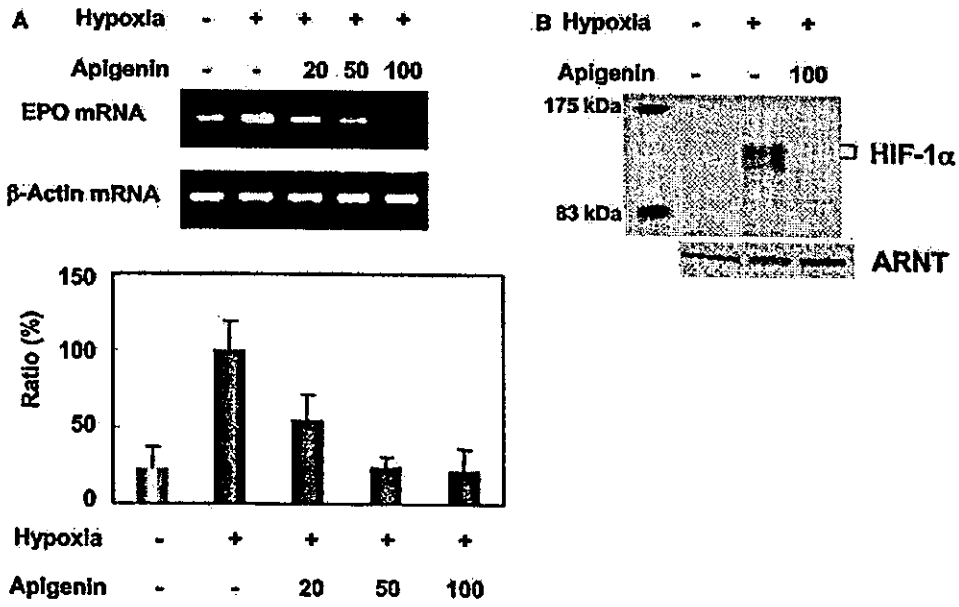

Fig. 2. Expression of EPO mRNA and HIF-1α in Hep3B cells under hypoxic conditions. (A) Hep3B cells were exposed to 5% O_2, 5% CO_2 and 90% N_2 for 3 h in the presence or absence of 20, 50, or 100 μM apigenin. EPO mRNA expression was analyzed by RT-PCR. The ratio (EPO mRNA/β-Actin mRNA) under hypoxia was 100%. Values are given as means ± S.D. for four separate experiments. (B) Hep3B cells were exposed to 5% O_2, 5% CO_2 and 90% N_2 for 3 hours in the presence or absence of 100 μM apigenin. Whole cell lysates were immunoprecipitated with anti-HIF-1α IgG and analyzed by SDS–PAGE with 7.5% poly-acrylamide gel, and then were immunoblotted with antibodies against HIF-1α. Whole cell lysates (50 μg) were analyzed by SDS–PAGE with 7.5% polyacrylamide gel and immunoblotted with anti-ARNT IgG. An ABC kit was used for the secondary antibody and HIF-1α was detected with 4-chloro-1-naphthol.

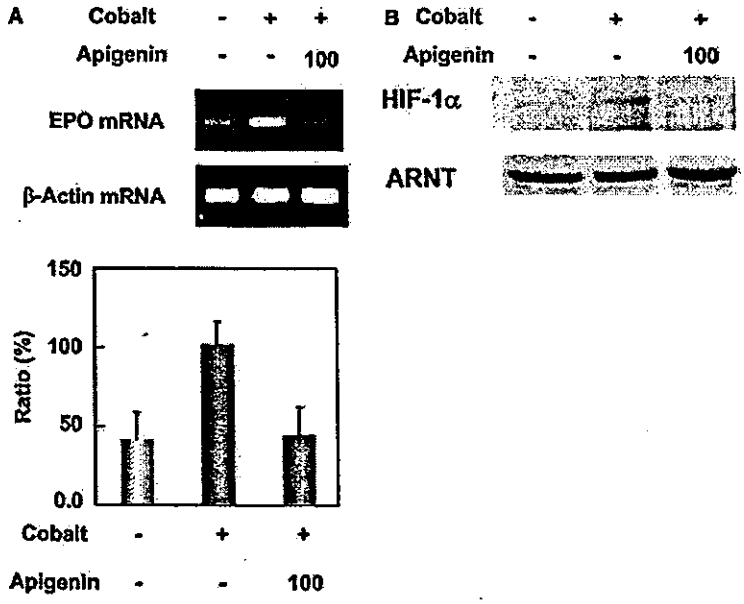

Fig. 3. Expression of EPO mRNA and HIF-1α in Hep3B cells treated with cobalt. Hep3B cells were exposed to 100 μM cobalt for 3 h in the presence or absence of 100 μM apigenin. (A) EPO mRNA expression was analyzed by RT-PCR. The ratio (EPO mRNA/β-Actin mRNA) under hypoxia was 100%. Values are given as means ± S.D. for four separate experiments. (B) Whole cell lysates were analyzed by SDS–PAGE with 7.5% poly-acrylamide gel and immunoblotted with antibodies against HIF-1α (1:100). An ABC kit was used for the secondary antibody and HIF-1α was detected with 4-chloro-1-naphthol.

We studied whether or not flavonoids other than apigenin have an inhibitory effect on the expression of EPO mRNA induced by hypoxia. We found that kaempferol and myricetin have an inhibitory effect on the expression under hypoxic conditions, but catechin and epicatechin do not (data not shown). Wilson et al. [31] demonstrated that the flavonoid quercetin stabilizes HIF-1α and induces the expression of VEGF mRNA under normoxic conditions. Moreover, it has been proposed that quercetin regulates the response to hypoxia by inhibiting the activity of HIF hydroxylase, a member of the Fe(II), 2-oxoglutarate-dependent dioxygenase family, because quercetin is a good iron chelator [32].

In this study, we found that apigenin reduced HIF-1α levels by interfering with the binding of HIF-1α to Hsp90, leading to a suppression of EPO mRNA transcription. The angiogenesis frequently observed in tumors would be suppressed by apigenin via a reduction of VEGF mRNA expression. Since it may suppress a number of genes induced by HIF-1α, it is likely that apigenin could be a potent inhibitor of angiogenesis. Our results clearly show that apigenin is potentially an important chemical for controlling HIF-1α levels.

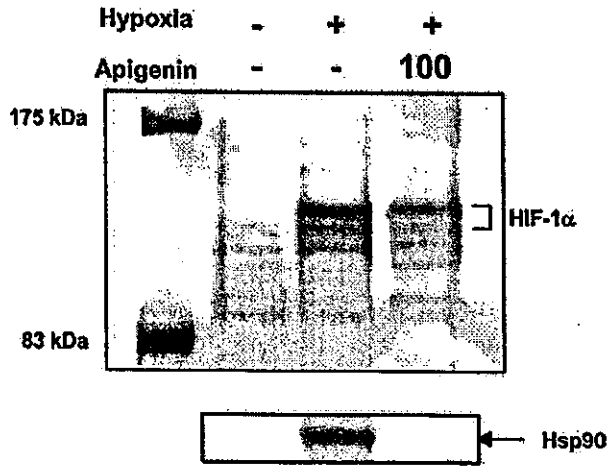

Fig. 4. Interaction of HIF-1α and Hsp90 proteins in the presence of apigenin. Hep3B cells were exposed to 5% O_2, 5% CO_2 and 90% N_2 for 3 h. Apigenin (100 μM) was added to whole cell lysates. The lysates were immunoprecipitated with anti-HIF-1α IgG and analyzed by SDS–PAGE with 7.5% polyacrylamide gel and immunoblotted with antibodies against HIF-1α (1:100) (A) and anti-Hsp90 IgG (B).

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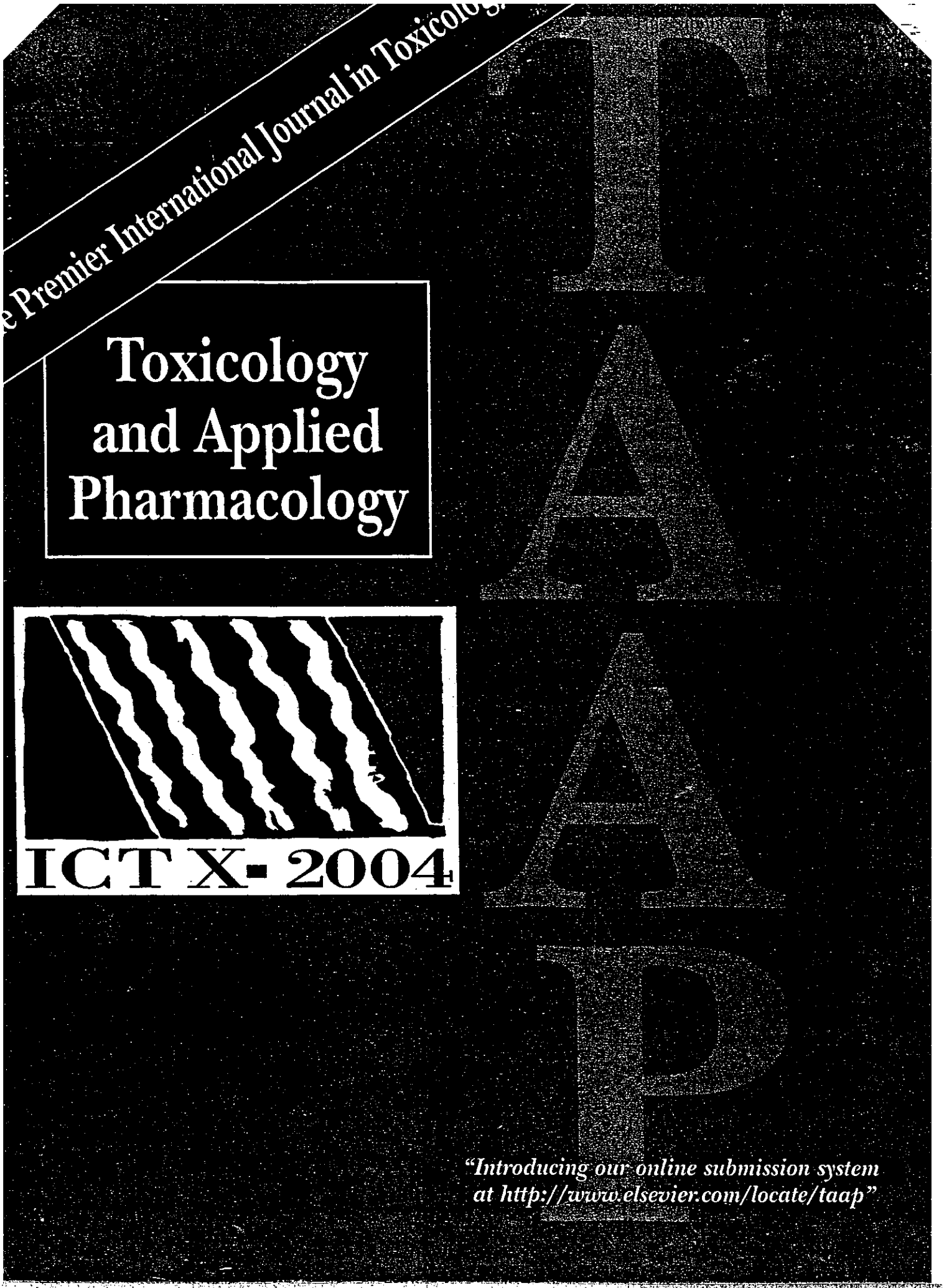
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