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＜総説＞

ストレス応答とステリルグルコシド

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1．はじめに

細胞は、高熱、浸透圧、酸化、重金属、紫外線など外界の様々な環境ストレスから自らを保護するために、熱ショックタンパク質（Hsp）を中心とするストレス防御機構をもっている。しかし、各種ストレスを最初に感知する実体、機構は何か、また、Hsp の誘導に先立つシグナル伝達系は何かについては不明の点が多い。この問題を解決する糸口として、我々は、ストレスを最初に感知する場である細胞膜に注目し、中でもその脂質成分の迅速な変動を解析した。その結果、ヒト由来の培養細胞に熱ストレスを与えると、ある種の糖脂質が特異的、かつ速やかに合成誘導されることを見出した。この分子を構造解析した結果、ステリルグルコシドの一種であるコレステリルグルコシド（Fig. 1）と同定された。

ステリルグルコシドは、種々のステロールにグルコースがα、あるいはβ-グルコシド結合した分子であり、高等植物に広く存在している[1,2]ほか、マイコプラズマ[3]、酵母[4]、真性粘菌[5,6]、ヘリコバクター[7-9]などにも存在する。ステロールやグルコースの輸送、膜融合などに関与する可能性や、グルコシルセラミド合成のグルコース供与体として働く可能性が示唆されているが、その生物学的・生理的機能についてはほとんど明らかにされていない。

本稿では、このステリルグルコシドの誘導が、細胞のストレス応答において積極的に関わっている可能性について、筆者らの最近の研究結果を基に考察したい。さらに、コレステリルグルコシドの経口投与がラットのストレス胃潰瘍の発症を抑制することを見出したので、その機構についても論じる。

2．熱ストレスによって誘導されるコレステリルグルコシド

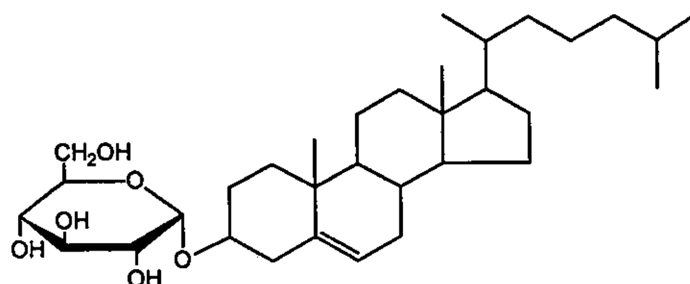
筆者らは、ヒト胎児由来正常繊維芽細胞（TIG-3 細胞）を用いて、ストレス依存的に変動する脂質成分について詳細に解析した。37℃で培養した細胞を、予め加温した培地に交換することによって 42℃に熱ショックを与え、その後も 42℃に保ちながら、経時的に細胞を回収して脂質抽出し、薄層クロマトグラフィーで分析した。その結果、熱ストレス後、15、および 30 分後に新たな脂質が検出され、その後 60 分後には消失した（Fig. 2）[10]。この未知脂質は、標準物質として用いた真性粘菌由来のステリルグルコシドであるポリフェラステリルモノグリコシド（PSMG）よりやや小さなRf値を示した。また、呈色反応からこの物質は糖とステロールを含む脂質と推定された。なお、この時のHsp70 は温度シフトしてから約 60 分後に誘導されており、この脂質の誘導はHsp70 に先立って起こる現象であった。

この未知脂質を精製して構造決定した結果、β-コレステリルグルコシド（Fig.1）であった。熱ストレス依存的なステリルグルコシドの合成誘導は真性粘菌*Physarum polycephalum*で初めて見出された現象[6]であるが、ヒト由来培養細胞においても確認されたことになる。最近、酵母の一種*Pichia pastoris*においても同様の報告[11]がされており、熱ストレス依存的なステリルグルコシドの誘導は生物界に普遍的な現象であると考えら

れる。

TIG-3 細胞の膜画分には、UDP-グルコースからコレステロールへグルコースを転移するグルコシルトランスフェラーゼ活性が存在することが示されており、この点、真性粘菌の場合[12]と類似している。ステリルグルコシドの合成酵素については植物での研究が進んでおり、ムギやシロイヌナズナのUDP-グルコース：ステロールグルコシルトランスフェラーゼ遺伝子がクローニングされ[13]、続いて酵母や細胞性粘菌でも同種の酵素がクローニングされた[14]。これらの配列には、植物、酵母、細胞性粘菌にわたって共通の保存領域が含まれており、UDP-糖転移酵素のスーパーファミリーであるUGT スーパーファミリーに共通の配列、ステロイド結合ドメインと考えられる領域などが予想されている。しかし、哺乳動物での遺伝子は、現在までのところ特定されていない。

(A)　α-コレステリルグルコシド（α-CG)



(B)　β-コレステリルグルコシド（β-CG)

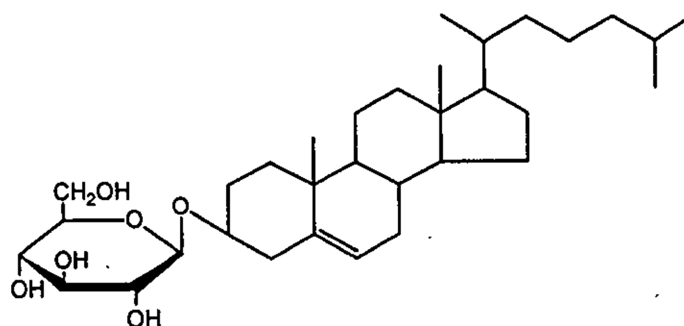


Fig. 1　コレステリルグルコシドの構造
(A) α-コレステリルグルコシドは *Acholeplasma*、*Helicobacter pylori* などに存在する。(B) β-コレステリルグルコシドはタバコ、*Candida*、ニワトリ上皮などの他、ヒト繊維芽細胞に存在する。

3．コレステリルグルコシドによる Hsp の誘導

前述したように、熱ストレスによるコレステリルグルコシドの蓄積は、Hsp70 の誘導される時間よりも早く起こることから、Hsp誘導に至るシグナル伝達の上流にステリルグルコシドが位置することが考えられる。そこで、TIG-3 細胞の培養上清に有機合成したコレステリルグルコシドを添加した場合に、熱ストレスをかけなくともHsp70 誘導が起こるかを検討した。その結果、熱ストレス処理時より早い時間でHsp70 の誘導が観察されたことから、コレステリルグルコシドは細胞の熱ストレス応答における初期の段階で作られ、その後のHsp誘導を惹起する重要な分子であることが示唆された[15]。

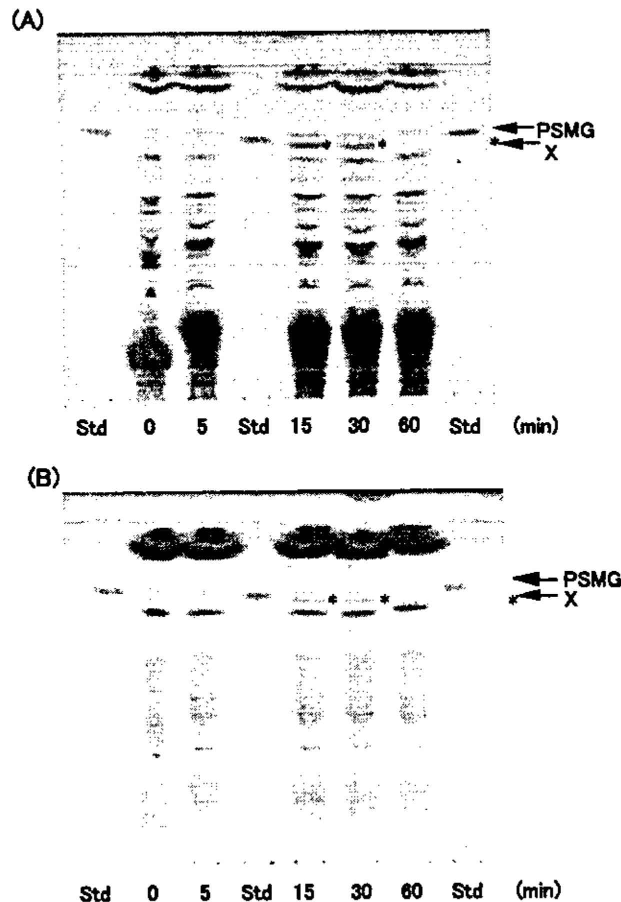


Fig. 2　ヒト正常繊維芽細胞（TIG-3）での熱ストレスによる脂質組成の変化[10]
37℃で培養したTIG-3細胞を42℃の培地に変換後、42℃で5、15、30、60分間インキュベートした。その後、脂質成分を抽出して薄層クロマトグラフィーを用いて分析した。展開溶媒はクロロホルム／メタノール／水（60:40:9）を使用した。(A) はオルシノール硫酸試薬による糖の発色、(B) は塩化第二鉄試薬によるステロールの発色を示す。PSMG；標準品（Std）として用いた真性粘菌由来のポリフェラステロールモノグルコシド。X;熱ストレスにより誘導されたステロール配糖体。

さらに筆者らは、ラットから摘出した胃粘膜細胞を初代培養して *in vitro* で熱ストレスを与えることにより、胃粘膜におけるストレス応答の解析を行った。その結果、ラット胃粘膜細胞においても TIG-3 細胞と同様に、熱ストレス依存的に速やかなコレステリルグルコシドの増加が観察され、引き続き Hsp70 の誘導が見られた。

以上の知見をもとに、ステリルグルコシドを介する細胞ストレス応答の分子機構について考察すると、Fig.3 に示したようなスキームが想像される。すなわち、細胞が熱ストレスなどに曝されると、細胞膜の物性変化を介して細胞膜のステリルグルコシド合成酵素が活性化されてステリルグルコシドが合成誘導される。蓄積したステリルグルコシドは、細胞内に存在するある種のタンパク質キナーゼを活性化するなどして、最終的に HSF1 をリン酸化して活性化し、HSE との結合を介して Hsp 遺伝子の発現を誘導する。細

胞内のタンパク質キナーゼの存在とその活性化機構など今後検討していくべき課題も多いが、ステリルグルコシドを中心とした新たな細胞内ストレス応答機構として注目される。

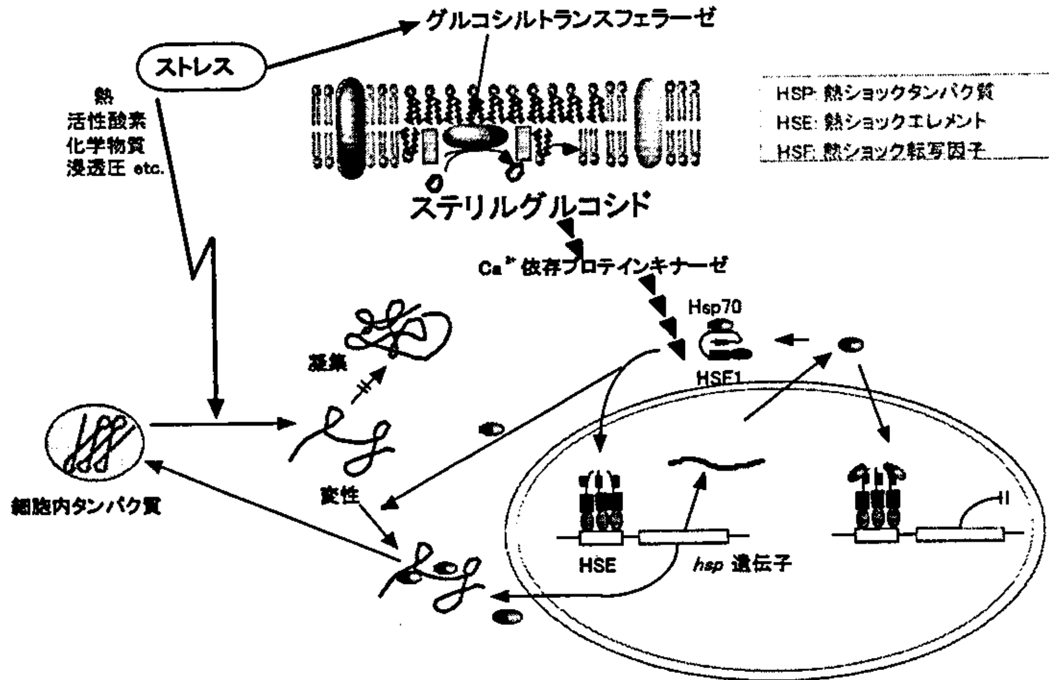


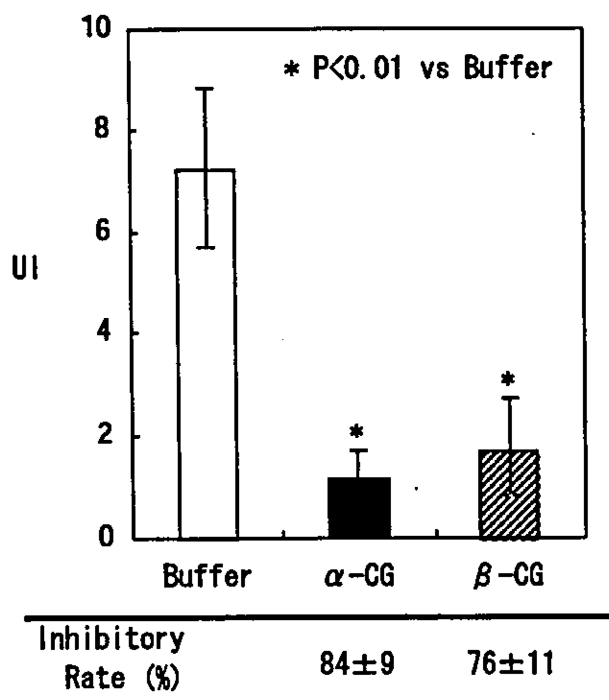
Fig. 3　ステリルグルコシドを介するストレス応答の分子機構（仮説）
ストレスを受けると、細胞膜に存在するグルコシルトランスフェラーゼが活性化され、ステリルグルコシドが合成される。ステリルグルコシドによりある種のプロテインキナーゼが活性化され、このキナーゼによって HSF1 がリン酸化、活性化されて Hsp 発現を誘導する。

4．コレステリルグルコシドの抗胃潰瘍作用

細胞内ストレス応答において、Hspはタンパク質変性を防ぐ分子シャペロンとして働き、その誘導は様々なストレスに対して細胞保護作用を示す。一方、拘束ストレスなどの個体レベルで受けるストレスによっても、内分泌系や自律神経系によるストレス反応を介して、最終的には細胞レベルでのストレス応答が惹起され、その結果、細胞内でHspが誘導されることが生体防御に役立っていると考えられている。Hspは、虚血・再灌流などによる酸化ストレス、低濃度アルコールなどの物理的・化学的傷害因子に対して抗アポトーシス作用を示す。また、高濃度のエタノールによる急性ネクローシスもHsp70 により抑制されることが示されている[16]。したがって、このHsp誘導を医薬品や食品分野へ効果的に利用することが期待される。ラットを用いた寒冷拘束ストレスによる消化器潰瘍の発症モデルでも、胃粘膜細胞での適度なHspの誘導が細胞保護作用を発揮して潰瘍症状の軽減化をもたらすと考えられており、現在、抗潰瘍薬として臨床的に使われているテプレノンは、その主要な作用機序としてHsp 誘導が報告されている[17]。胃の血流調節や胃

粘膜の保護に働く内因性物質としては、従来よりプロスタノイドやNOが知られていた。しかし、プロスタグランジンシンターゼIまたはIIを欠損したマウスにおいて胃粘膜傷害が増進しなかったことから、適応性の細胞保護は単一の因子によるものだけでは説明できないと考えられる。したがって、プロスタグランジン以外の細胞保護作用を示す因子を明らかにすることは、胃粘膜におけるストレス応答の解明にとって重要であると考えられる。筆者らが見出したコレステリルグルコシドはその有力な候補であり、Hspの誘導を介して胃潰瘍の発症と深く関わっていると考えられる。

ラット寒冷拘束ストレス潰瘍モデルを用いて解析した結果、以下に述べるように、コレステリルグルコシドの経口投与が抗潰瘍作用を示すことが明らかとなった[18]。コレステリルグルコシドを経口投与し、30分間放置したラットに寒冷拘束ストレスを与え、胃粘膜の様子を観察した。コントロール群として、Bufferのみを投与したラットの胃粘膜では、寒冷拘束ストレス誘導性の胃潰瘍が胃粘膜全体にわたって観察され、出血性の潰瘍もみられた。一方、100mg/kgのコレステリルグルコシド投与群では、α、あるいはβ-型ともにBuffer群に比べ潰瘍の数も少なく、その大きさも小さかった。Fig.4 に、各群における潰瘍の長さの総和 (Ulcer Index, UI) をプロットした図を示す。潰瘍形成の抑制率は、α-コレステリルグルコシド投与群では84±9%、β-コレステリルグルコシド投与群では76±11%であり、有意な抑制作用を示した。



Inhibitory Rate (%)		84±9	76±11

Fig. 4　寒冷拘束ストレス誘導性潰瘍に対するコレステリルグルコシドの影響（文献18より改変）

各群（n=5）のラット胃粘膜における潰瘍の程度。UI は胃壁での潰瘍部の長さの総和を示す。Buffer:バッファーのみ前投与後にストレスを与えたラット群、α- CG、β-CG：α-、またはβ-コレステリルグルコシドを経口にて前投与後、寒冷拘束ストレスを与えたラット群。数値は、平均±標準誤差を表す。

この作用機序を明らかにするために、コレステリルグルコシドを投与したラットの胃粘膜におけるHsp70の誘導を詳しく解析した。その結果、HSF1-HSEの結合活性が投与後5分以内に起こり、続いてHsp70 mRNAが発現され、続いてHsp70タンパク質の合成誘導が起こることが示された[18]。また、Hsp70誘導の時間的推移を比較すると、コレステリルグルコシドによる誘導は、熱ストレスと比べ速い応答であったため、コレステリルグルコシドが生体内で合成されてHsp70誘導のシグナルとして働いている可能性が示唆された。

生体が個体レベルのストレスを受けると、視床下部-下垂体-副腎皮質系（HPA system）によるグルココルチコイド分泌と、視床下部-交感神経-副腎髄質系（SAM system）によるアドレナリン分泌を介して胃粘膜において胃酸分泌亢進、胃粘膜分泌減少、平滑筋・血管収縮とそれにともなう血流障害が起こる。このような胃粘膜での環境変化に応答して、胃粘膜細胞でのコレステリルグルコシド誘導が惹起されてHsp70の誘導を引き起こし、このHspによる抗アポトーシス、または抗ネクローシス作用により胃粘膜細胞の保護作用を示すと考えられる（Fig.5)。以上から、コレステリルグルコシドはストレス時に細胞内で合成誘導される生理活性物質であることに加え、Hsp誘導剤として胃粘膜傷害を軽減化する医薬品として利用できる可能性が示された。

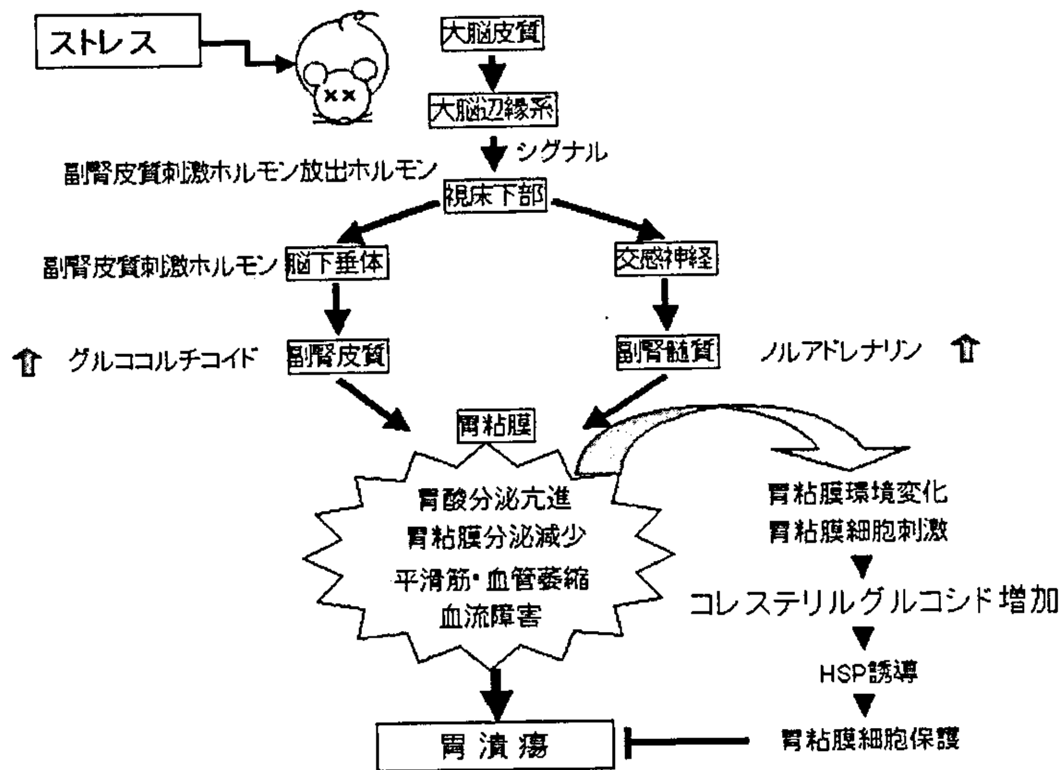


Fig.5　コレステリルグルコシドによるHSP誘導を介したラット胃潰瘍の抑制機構

5．ステリルグルコシドのもつその他の生理機能

ステリルグルコシドの生体内機能について、これまであまり多くの報告はない。植物では、膜の主要構成成分としてグルコースやステロールの輸送への関与[5, 6, 19]、グルコシルセラミド合成のグルコース供与体として働く可能性[20]などが考えられている。最近では、疾患治療に応用可能な有益な情報が、植物由来のステリルグルコシドについて少しずつ報告されはじめている。Yasukawaらは、12-*O*-tetradecanoyl-phorbol-13-acetate (TPA) による腫瘍誘導作用を、乾燥薬草の一種 (*Euphorbia kansui*) の根由来のトリテルペンアルコールとβ-シトステリルグルコシドが抑制することを示した[21]。また、Rubnov らは、イチジク (*Ficus carica*)の樹脂由来の 6-*O*-acyl-beta-D-glucosyl-beta-sitosterol (アシル型のシトステリルグルコシド) を含む成分に、ガン細胞増殖抑制能がみられたことを報告している[22]。また、栄養学的な面でも注目されており、β-シトステリルグルコシド摂取により、T細胞の一種であるNK細胞の活性が高まることが報告されている[23]。さらに、植物由来のステリルグルコシドがウサギ鼻粘膜での薬剤吸収を促進すること[24]、ステリルグルコシドを含むリポソームが肝臓へのドラッグキャリアー[25]や遺伝子ターゲティングに有用であること[26]なども示され、ステリルグルコシドの新たな機能が注目されている。

6．おわりに

本稿では、ステリルグルコシドの生理機能について、細胞のストレス応答との関係を中心に最近の知見をまとめた。哺乳動物細胞において、ステリルグルコシドはHsp誘導のためのメディエーター様分子として機能している可能性が考えられること、また、その経口投与はHspの誘導を介して抗胃潰瘍作用を発揮することが動物実験で示されたことなどを紹介した。胃潰瘍や胃がんなどとの関連が指摘されている病原菌として知られているヘリコバクター・ピロリには、α-コレステリルグルコシドを骨格としたユニークなステリルグルコシドが豊富に存在することが報告されている[7-9]。これらのステリルグルコシドが、宿主の胃上皮細胞のもつステリルグルコシドを中心としたストレス応答系を撹乱して細胞傷害を示している可能性も考えられ、今後のさらなる解析が期待される。また、Hspが関与する病態には、消化器潰瘍以外にも、癌、嚢胞性繊維症、自己免疫疾患、アルツハイマー病やハンチントン症など、様々なものが知られている。ステリルグルコシドは、細胞内Hsp発現レベルの調節を通して、これら疾患の予防や治療に役立つことが考えられる。ステリルグルコシドは、大豆などの食品にも多く含まれる成分であり、機能性食品成分としても注目されるであろう。

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Note

Oxysterol Regulation of Estrogen Receptor α-Mediated Gene Expression in a Transcriptional Activation Assay System Using HeLa Cells

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In order to test the estrogenic activity of sterol oxidation products from cholesterol and phytosterols, an estrogen-dependent gene expression assay was performed in estrogen receptor α-stably transformed HeLa cells. The ranking of the estrogenic potency of these compounds was different: 17β-estradiol ≫ genistein ≫ β-epoxycholesterol = daidzein = cholestanetriol = 22(R)-hydroxycholesterol = 20(S)-hydroxycholesterol = sitostanetriol > campestanetriol = β-epoxysitosterol = 7β-hydroxycholesterol. These compounds were not estrogenic in estrogen receptor-negative HeLa cells.

Key words: oxysterols; estrogen receptor; cholesterol; phytosterol; phytoestrogen

Phytoestrogens such as genistein and daidzein, and endocrine disrupters such as bisphenol A and *p-n*-nonylphenol, have estrogenic activities.[1] Although cholesterol is not estrogenic, β-sitosterol, one of the most abundant phytosterols in nature, in which the side chain structure differs slightly from that of cholesterol, has an estrogenic potency *in vivo*[2] and *in vitro*.[3] In recent years, sterol oxidation products (oxysterols) from cholesterol have attracted wide attention as activators for liver X receptor (LXR), one of the nuclear receptors.[4] The overall structure of the LXR ligand binding domain (LBD) is similar to that seen in the crystal structure of other nuclear receptor LBDs.[5] Estrogen receptor (ER) is one such nuclear receptor. In this study, we examined the effects of oxysterols from cholesterol and phytosterols on estrogenic potential in a transient gene expression assay using human ERα-stably transformed HeLa cells. Our results indicate that some oxysterols from cholesterol and phytosterols stimulate ERα-mediated gene expression.

Cholesterol-derived oxysterols such as cholestan-5α,6α-epoxy-3β-ol (α-epoxycholesterol), cholestan-5β,6β-epoxy-3β-ol (β-epoxycholesterol), cholest-5-en-3β-ol-7-one (7-ketocholesterol), cholest-5-en-3β,7α-diol (7α-hydroxycholesterol), cholest-5-en-3β,7β-diol (7β-hydroxycholesterol), cholest-5-en-3β,20(S)-diol [20(S)-hydroxycholesterol], cholest-5-en-3β,22(R)-diol [22(R)-hydroxycholesterol], cholest-5-en-3β,22(S)-diol [22(S)-hydroxycholesterol], cholest-5-en-3β,25-diol (25-hydroxycholesterol), 25R-cholest-5-en-3β,26-diol (27-hydroxycholesterol), and cholestan-3β,5α,6β-triol (cholestanetriol) were purchased from Steraloids Inc. (Wilton, New Hampshire, U.S.A.). β-Sitosterol, campesterol, and the phytosterol mixture (β-sitosterol 52.5%, stigmasterol 2.0%, campesterol 45.5%) that were obtained from Tama Biochemical (Tokyo, Japan) were used as starting materials for preparing phytosterol-derived oxysterols such as campestan-5α,6α-epoxy-3β-ol (α-epoxycampesterol), campestan-5β,6β-epoxy-3β-ol (β-epoxycampesterol), β-sitostan-5α,6α-epoxy-3β-ol (α-epoxysitosterol), β-sitostan-5β,6β-epoxy-3β-ol (β-epoxysitosterol), campestan-3β,5α,6β-triol (campestanetriol), and β-sitostan-3β,5α,6β-triol (sitostanetriol), as described previously.[6] Cholesterol and 17β-estradiol were purchased from Sigma (St. Louis, Missouri, U.S.A.). Soy isoflavones (genistein, daidzein, and glycitein) were obtained from Fujikko (Kobe, Japan). Citrus-derived products (hesperetin and limonin) and endocrine disrupters (bisphenol A and *p-n*-nonylphenol) were purchased from Wako Pure Chemical (Osaka, Japan).

pCI-neo-ERα vector was constructed by insertion of human ERα cDNA derived from pIC-ER-F (ATCC, Manassas, Virginia, U.S.A.) in pCI-neo mammalian expression and neomycin resistant vector (Promega, Madison, Wisconsin, U.S.A.). pGL3-estrogen responsive element (ERE) luciferase reporter vector, pGL3-ERE, was constructed by insertion of four copies of *Xenopus* vitellogenin ERE[7] into pGL3-Basic vector (Promega). *Renilla* luciferase constitutive expression vector pRL-CMV was obtained from Promega for estimating transfection efficiency. HeLa cells that were obtained from Riken Gene Bank (Tokyo, Japan) were plated and cultured at 90% confluence in minimum

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Abbreviations: oxysterols, sterol oxidation products; LXR, liver X receptor; LBD, ligand binding domain; ER, estrogen receptor; ERE, estrogen responsive element; MEM, minimum essential medium; FBS, fetal bovine serum; PBS (−), phosphate-buffered saline without Ca^{2+} or Mg^{2+}

essential medium (MEM, Invitrogen, Carlsbad, California, U.S.A.) containing 10% fetal bovine serum (FBS, Thermotrace, Melbourne, Australia) in an atmosphere of 5% CO_2 at 37 °C. The cells were washed once with Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (PBS (−), Nissui Pharmaceuticals, Tokyo, Japan). The cells were transfected with 5 μg of pCI-neo-ERα with LIPOFECTAMINE reagent (Invitrogen) and PLUS reagent (Invitrogen) according to the manufacturer's instructions. After 48 h, the medium was changed to MEM supplemented with 10% FBS and 400 μg/ml of antibiotic G418 (Nacalai Tesque, Kyoto, Japan) for selection. After one month, the vector stably transformed (G418 resistant) cells were grown as colonies. The cloned cells were assayed for estrogen responsiveness by luciferase assay, as described below. The responsive colonies were cloned further by limited dilution for purification. This clone was termed ERα-HeLa.

ERα mRNA expression in ERα-HeLa cells was measured by northern blot-hybridization analysis and compared with HeLa cells that were transiently transfected with 5 μg of pCI-neo-ERα or pCI-neo vector in 10 cm dishes.[8] The cellular total RNAs were isolated by acid guanidium thiocyanate-phenol-chloroform extraction.[9] 20 μg of cellular total RNA were electrophoresed in individual lanes.

The ERα-HeLa cells were seeded at a concentration of 5×10^4 cells per well in 24-well plates and incubated for 24 h in phenol red-free MEM containing 10% charcoal/dextran-treated FBS (Hyclone, Logan, Utah, U.S.A.). The medium was replaced with phenol red-free MEM containing 5% charcoal/dextran-treated FBS. After 24 h, the cells were transfected with 310 ng of pGL3-ERE and 31 ng of internal control pRL-CMV vector by LIPOFECTAMINE reagent and PLUS reagent in individual wells. After 24 h, the cells were rinsed with PBS (−) and the medium was replaced with phenol red-free MEM containing 5% charcoal/dextran-treated FBS and a desired concentration of test chemicals that were dissolved in ethanol. The final ethanol concentration was 0.1%. Following 24 h incubation, the cells were rinsed with PBS (−) and were harvested in Passive Lysis Buffer (Promega). Luciferase assay in HeLa cells was performed as in the case of the ERα-HeLa cells except for transfection with 31 ng of pCI-neo or pCI-neo-ERα, 310 ng of pGL3-ERE, and 31 ng of pRL-CMV. The firefly and *Renilla* luciferase activities of the cell lysates were measured with the Dual-Luciferase Reporter Assay System (Promega) with Lumat LB 9507 luminometer (EG&G Berthold, Bad Wildbad, Germany). Statistical analysis was performed by Dunnett's multiple comparison test or Student's *t*-test.

Figure 1 shows the results of northern blot-hybridization analysis on ERα. ERα mRNA was detected in ERα-HeLa cells strongly and pCI-neo-ERα-transiently transfected HeLa cells intermediately. But ERα mRNA was not detected in pCI-neo-transiently transfected HeLa cells.

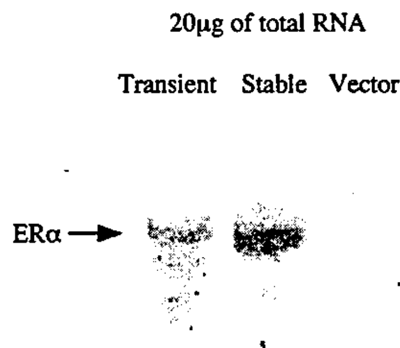

Fig. 1. Northern Blot-hybridization Analysis of ERα.

Total RNAs derived from ERα-HeLa (Stable) and HeLa cells that were transiently transfected with pCI-neo-ERα (Transient, as positive control) or pCI-neo (Vector, as negative control) vector were analysed by northern blotting. 20 μg of total RNAs were electrophoresed in individual lanes.

As shown in Table 1, the relative transcriptional activity of each compound was calculated as the ratio of the luciferase reporter gene induction value of each compound to that of the no additive (0.1% ethanol) group. 17β-Estradiol and genistein stimulated specific reporter gene activity at concentrations of 10 nM and 1 μM respectively. As for other compounds, however, including sterols, oxysterols, endocrine disruptors, citrus products, and soy isoflavones, estrogenic activity was not seen below a concentration of 1 μM. Then, estrogenic activity was investigated at the concentration of 10 μM. Neither cholesterol nor the phytosterol mixture showed estrogenic action. On the other hand, some oxysterols derived from these sterols showed estrogenic activity. The ranking of the estrogenic potency of these compounds for ERα in the transactivation assay was as follows: 17β -estradiol ≫ genistein ≫ hesperetin = β-epoxycholesterol = daidzein = cholestanetriol = 22(R)-hydroxycholesterol = 20(S)-hydroxycholesterol = sitostanetriol > campestanetriol = β-epoxysitosterol = 7β-hydroxycholesterol. The remainder of the tested compounds resulted in no estrogenic activity. Two endocrine disrupters, bisphenol A and *p*-*n*-nonylphenol, resulted in no estrogenic action. One of the citrus-derived products, hesperetin, caused a significant elevation of estrogenic activity to the same extent as daidzein. This finding is in accordance with a report that hesperetin was estrogenic in human breast cancer MCF-7 cells.[10]

It is interesting to note that 22(R)-hydroxycholesterol was estrogenic, but it was not so for the enantiomer, 22(S)-hydroxycholesterol in Table 1. It has been reported that although 22(R)-hydroxycholesterol binds in the core of the LBD of LXRα as the agonist, 22(S)-hydroxycholesterol does not.[4] Hence, it is expected that some oxysterols of animal or plant origin can combine with the LBD of ERα, thereby promoting estrogenic activity. In order to elucidate the mechanism of estrogenic activity by oxysterol, we performed a luciferase assay on the estrogenic action of typical oxysterols in

Table 1. Relative Transcriptional Activity of Oxysterols and Various Chemicals for ERα

Chemical	Fold activation	Chemical	Fold activation
No additive (ehtanol)	1.00 ± 0.10^{b}	*Oxidized phytosterols*	
17β-Estradiol	9.51 ± 0.57	α-Epoxycampesterol	0.98 ± 0.22^{b}
Sterols		α-Epoxysitosterol	0.83 ± 0.25^{b}
Cholesterol	0.96 ± 0.06^{b}	β-Epoxycampesterol	0.66 ± 0.19^{b}
Phytosterol mixture	1.50 ± 0.20^{b}	β-Epoxysitosterol	2.56 ± 0.44^{ab}
Oxidized cholesterols		Campestanetriol	2.91 ± 0.45^{ab}
α-Epoxycholesterol	1.62 ± 0.11^{b}	Sitostanetriol	3.50 ± 0.17^{a}
β-Epoxycholesterol	4.84 ± 0.39^{a}	*Endocrine disrupting chemicals*	
7-Ketocholesterol	1.44 ± 0.10^{b}	Bisphenol A	1.24 ± 0.15^{b}
7α-Hydroxycholesterol	1.69 ± 0.05^{b}	*p-n*-Nonylphenol	1.76 ± 0.18^{b}
7β-Hydroxycholesterol	2.52 ± 0.18^{ab}	*Citrus-derived products*	
20(S)-Hydroxycholesterol	3.60 ± 0.16^{a}	Limonin	1.87 ± 0.30^{b}
22(R)-Hydroxycholesterol	3.87 ± 0.41^{a}	Hesperetin	5.30 ± 0.75^{a}
22(S)-Hydroxycholesterol	1.06 ± 0.04^{b}	*Soy isoflavones*	
25-Hydroxycholesterol	1.44 ± 0.12^{b}	Glycitein	1.23 ± 0.37^{b}
27-Hydroxycholesterol	1.61 ± 0.17^{b}	Daidzein	4.31 ± 0.27^{a}
Cholestanetriol	4.27 ± 0.60^{a}	Genistein	11.98 ± 1.71

ERα-HeLa cells were transfected with pGL3-ERE reporter vector and internal control pRL-CMV vector. After 24 h, the cells were treated with oxysterols and other chemicals for 24 h. All chemicals were added at 10 μM, except for genistein (1 μM) and 17β-estradiol (10 nM). The transcriptional activity of the no additive (0.1% ethanol) group was arbitrarily set as 1. Data are means ± SE for four determinations. The significance of the difference in the mean values other than those for genistein and 17β-estradiol was evaluated by Dunnett's multiple comparison test. ^{a}: $P < 0.01$ against the no additive group. ^{b}: $P < 0.01$ against the daidzein group.

Table 2. Oxysterol and Estrogen Did Not Activate ERα-Negative HeLa Cells

Chemical	ERα-negative	ERα-positive
No additive (ethanol)	1.00 ± 0.04	1.00 ± 0.08
β-Epoxycholesterol	0.98 ± 0.09	$1.48 \pm 0.10^{**}$
20(S)-Hydroxycholesterol	0.97 ± 0.09	$1.22 \pm 0.05^{*}$
22(R)-Hydroxycholesterol	0.98 ± 0.07	$1.58 \pm 0.04^{***}$
17β-Estradiol	1.06 ± 0.03	$2.61 \pm 0.14^{***}$

HeLa cells were transfected with pCI-neo (for ERα-negative) or pCI-neo-ERα (for ERα-positive) mammalian expression vector, pGL3-ERE reporter vector, and internal control pRL-CMV vector. After 24 h, the cells were treated with oxysterols (10 μM) and 17β-estradiol (10 nM) for 24 h. The transcriptional activity of the no additive (0.1% ethanol) group was arbitrarily set as 1. Data are means ± SE for four determinations. The significance of the difference in the mean values was evaluated by Student's *t*-test. *,**,***: $P < 0.05$, 0.01, or 0.001 against the same chemical-added ERα-negative group respectively.

ERα-expressing or non-expressing HeLa cells. As shown in Table 2, β-epoxycholesterol, 20(S)-hydroxycholesterol, 22(R)-hydroxycholesterol, and 17β-estradiol showed estrogenic effects in HeLa cells expressing ERα (ERα-positive), but they did not so in ERα-negative HeLa cells. Thus, at least, it was revealed that the estrogenic activity of oxysterol is mediated by ERα. It has been shown that these oxysterols are efficiently absorbed by laboratory animals.[11,12] Hence, it is important to examine further whether oxysterols derived from both cholesterol and phytosterols take a role in triggering many of the biological responses *in vivo.*

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Phytosterol Oxidation Products Are Absorbed in the Intestinal Lymphatics in Rats but Do Not Accelerate Atherosclerosis in Apolipoprotein E–Deficient Mice[1]

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ABSTRACT Phytosterol oxidation products (oxyphytosterols) are formed during the processing and storage of foods. However, it is unknown whether oxyphytosterols affect human health. To address these issues, we prepared β-sitosterol and campesterol oxides, evaluated their lymphatic absorption in rats, and examined the effect of an oxyphytosterol diet on atherosclerosis in apolipoprotein (apo) E–deficient mice. The lymphatic absorption of cholesterol and 6 oxyphytosterols (7α-hydroxy, 7β-hydroxy, β-epoxy, α-epoxy, dihydroxy, and 7-keto) of β-sitosterol or campesterol was assessed in thoracic duct–cannulated rats fed an AIN-93G–based diet containing 2.5 g of cholesterol, oxyphytosterols, or intact phytosterols per kg. Lymphatic recoveries (on a mass basis) of oxycampesterols (15.9 ± 2.8%, $n = 10$) and oxysitosterols (9.12 ± 1.77%, $n = 10$) were higher than for campesterol (5.47 ± 1.02%, $n = 12$, $P < 0.05$) and β-sitosterol (2.16 ± 0.37%, $n = 12$, $P < 0.05$), but lower than for cholesterol (37.3 ± 8.3%, $n = 6$, $P < 0.05$). Apo E–deficient mice were fed an AIN-93G–based diet containing 0.2 g oxyphytosterols or intact phytosterols per kg for 9 wk. Diet-derived oxyphytosterols accumulated in the serum, liver, and aorta. Furthermore, the oxyphytosterol diet increased oxycholesterol in the serum compared to the phytosterol diet. However, there was no significant difference between the 2 groups in the serum and aortic cholesterol concentration, the lesion area in the aortic root, or 8-iso-prostaglandin F2α concentration in the urine. These results indicate that exogenous oxyphytosterols are well-absorbed and accumulate in the body, but do not promote the development of atherosclerosis in apo E–deficient mice. J. Nutr. 134: 1690–1696, 2004.

KEY WORDS: • *phytosterol* • *oxyphytosterol* • *absorption* • *atherosclerosis*

Cholesterol oxidation products (oxycholesterols) are formed during the processing and storage of foods (1). Oxycholesterols are found in human plasma chylomicrons (2), indicating that they are absorbed from the digestive tract. Furthermore, Staprans et al. (3) showed that when a meal containing 400 mg cholestan-5α, 6α-epoxy-3β-ol (α-epoxycholesterol) is consumed by humans, α-epoxycholesterol in the serum is present in chylomicrons, chylomicron remnants, and endogenous lipoproteins (VLDL, LDL, and HDL). There are conflicting reports on the role of exogenous oxycholesterols in the development of atherosclerosis in experimental animals; although atherosclerosis was observed in some studies, it was not in others (4,5).

Some investigators found plant sterol oxidation products (oxyphytosterols) in foods (6–9) and in the serum from phytosterolemic patients (10), although there are fewer reports than for oxycholesterols. The oxides of β-sitosterol and campesterol cause cellular damage in cultured mouse macrophages similar to that caused by oxycholesterols (11). However, it is unknown whether oxyphytosterols affect human health.

To address these issues, we prepared β-sitosterol and campesterol oxides and evaluated their lymphatic absorption in rats with permanent cannulation of the thoracic duct (12). Then we examined the effect of an oxyphytosterol diet on atherosclerosis lesion size in apolipoprotein (apo)[3] E–deficient mice, as well as the level of oxyphytosterols in the serum, liver, and aorta and oxycholesterols in the serum. The level of 8-iso-prostaglandin F2α (8-iso-PGF2α) in the urine was also measured as an index of in vivo peroxidative stress.

MATERIALS AND METHODS

Materials. Highly purified campesterol (97.8% on a weight basis) and β-sitosterol (98.6% on a weight basis) were obtained from Tama Seikagaku and used as a starting material for the synthesis of [I] epimeric 7-hydroxyphytosterols and 7-ketophytosterols (13), [II] 5α,6α- and 5β,6β-epoxyphytosterols (14), [III] 5,6-dihydroxyphytosterols (15), and [IV] β-epoxyphytosterols (16). They were also used as

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[3] Abbreviations used: ACAT, acyl-coenzyme A:cholesterol acyltransferase; apo, apolipoprotein; GC/NICI-MS, GC negative ion chemical ionization mass spectrometry; 8-iso-PGF2α, 8-iso-prostaglandin F2α; LXR, liver X receptor.

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standards for quantifying oxysterols by GC/MS in diets and animal tissues. Alternatively, a phytosterol mixture (Merck-Japan) composed (on a weight basis) of 37.8% campesterol, 54.3% β-sitosterol, 3.4% stigmasterol, 0.5% campestanol, 1.3% sitostanol, and 2.7% unknown compounds was used as a starting material for the preparation of oxyphytosterols as described above (13–16), an equal amount of the synthesized oxyphytosterols was mixed, and the mixture was employed in Study 1 (Table 1). Because the methods used (13–16) for the synthesis of oxyphytosterols produced unknown compounds other than the target compound to different extents, the amount of individual target oxyphytosterols shown in Table 1 differed. In another experiment, the phytosterol mixture was heated at 150°C for 12 h. Then the heated products were applied to a silicic acid column and the resultant oxyphytosterols were eluted with acetone after being washed with diethyl ether as previously described (17). The heat-prepared oxysterols were then used in Study 2 (Table 1).

Animals and diets. Animals were individually housed in a temperature-controlled room at 22–25°C with a 12-h light-dark cycle (lights on 0800–2000 h). All diets were based on the AIN-93G diet formulation (18), as previously described (19). Experiments were carried out under the Guidelines for Animal Experiments at the Faculty of Agriculture and the Graduate Course, Kyushu University, Fukuoka, Japan, and Law No. 105 and Notification (No. 6) of the Government of Japan.

In Study 1, 8-wk-old male Sprague-Dawley rats were obtained from Seiwa Experimental Animals. They were trained to consume a basal diet containing 100 g/kg lard as dietary fat twice per day from 1000–1100 and 1600–1700 h, respectively, for 1 wk. Lard was chosen to avoid an effect of phytosterols derived from dietary oils on the lymphatic transport of the phytosterols in rats because it contains only a small amount of phytosterols (7.2 mg campesterol and 8.1 mg β-sitosterol per kg lard). Deionized water was freely available throughout the feeding periods. All rats were anesthetized with Nembutal prior to permanent cannulation of the thoracic duct according to an original method (12) modified by us (20,21). Briefly, a cannula (Silicon tube SH, i.d. 0.5 mm and o.d. 1.0 mm, product of Kaneka Medics) filled with heparinized saline was inserted into the thoracic duct and secured within the abdominal cavity. The rats were returned to their cages and provided with the basal diet twice per day. On d 3, the rats were attached to a long PE-cannula (i.d. 0.58 mm and o.d. 0.97 mm, Becton-Dickinson) to collect the lymph. The end of the cannula was 5–10 cm below the bottom of the cage to provide sufficient underside pressure to allow the lymph to enter the cannula. The lymph was collected for 15 min in a tube containing 50 μg BHT and the rats were then given free access to the diet (g/kg diet) supplemented with 2.5 cholesterol (control diet), 2.5 cholesterol plus 2.5 phytosterol mixture from Merck-Japan (phytosterol diet), or 2.5 cholesterol plus 2.5 oxyphytosterol (oxyphytosterol diet) (Table 1) for 30 min. This amount of oxycholesterols was used to evaluate the absorption (21), because we wanted to compare the absorption rate of phytosterols and oxyphytosterols with that of cholesterol. At this time, the lymph was collected every hour for 7 h. The rats freely consumed deionized water during lymph collection. After fibrin was removed, 268 nmol/L EDTA was added to the lymph solutions. The solution was flushed with argon (purity 99.9% on a volume, Hakata Kyoudou Sannso) and stored at −30°C for up to 12 h. Apparent lymphatic recovery (% on a mass) for 7 h worth of sterols (oxyphytosterols, phytosterols, or cholesterol) was calculated according to the formula [(lymphatic transport of sterols for 7 h after meal) − 4 × 7 × (lymphatic transport of sterols for 15 min before meal)]/(amount of sterols consumed for 30 min). Transport of sterols in the lymph collected for 7 h after the meal was first deducted from the values that were acquired by converting the transport of sterols in the basal lymph collected for 15 min to that of 7 h and then divided by the amount of sterols consumed for 30 min. The transport of sterols for 7 h in the basal lymph was estimated by multiplying the transport of sterols for 15 min by 28 (4 × 7).

In Study 2, apo E–deficient mice purchased from Jackson Laboratories in 1994 were used (19). Male apo E–deficient mice (7–11 wk old) with an initial weight of 25.2 ± 0.6 g were divided into 2 groups, and the mice were fed a basal diet containing 100 g/kg palm oil as the dietary fat supplemented with 0.2 g/kg phytosterol mixture from Merck-Japan (phytosterol diet) or 0.2 g/kg heat-prepared oxyphytosterols (oxyphytosterol diet) (Table 1). This amount of oxycholesterols was used previously to evaluate their effect on atherosclerosis in mice (17). Palm oil was chosen to avoid an effect of cholesterol derived from dietary oils on the development of atherosclerosis in apo E–deficient mice because it is a dietary fat which contains negligible amounts of cholesterol. Experimental diets were packed in a pouch containing an O_2 absorbent (Ageless S-200; Mitsubishi Gas Chemical), flushed with N_2 and stored at 4°C. The diet was freshly prepared every week and changed every 2 d. Any remaining diet was discarded. At the end of the 9-wk feeding period, the mice were deprived of food for 4 h prior to killing. During the week before killing, mice were put in a metabolic cage (Shinano Seisakusho) where they freely consumed the diet and water, followed by urine collection in a container containing BHT (final concentration of 453 nmol/L) for 24 h. The urine was frozen in liquid nitrogen after Ar was blown through it and kept at −85°C. At the end of the experiment, the mice were anesthetized by intraperitoneal injection of sodium pentobarbital (50 mg/kg body wt) and killed by withdrawing blood from the left ventricle. The blood was transferred into 1-mL microcentrifuge tubes containing 50 μg BHT. The serum was separated, bubbled with Ar, and stored at −85°C after being frozen in liquid nitrogen. Livers and aortas were immediately removed from the carcasses, frozen in liquid nitrogen, and stored at −85°C. Prior to freezing, adipose tissue around the aorta was dissected away, rinsed in fresh PBS, and blotted dry between filter paper.

Determination of sterols and oxysterols. Oxysterols were measured using the method used for oxycholesterol (17,21). To 200 μL of serum and 0.25 g of liver was added 100 μg of 5α-cholestane (Sigma) and 1 μg of 19-hydroxycholesterol (Steraloids) as an internal standard. Then 200 μL of lymph was added to 50 μg of 5α-cholestane and 1 μg of 19-hydroxycholesterol. The total aorta was combined with 10 μg of 5α-cholestane and 1 μg of 19-hydroxycholesterol. Lipids in the samples were extracted with 20 vol of chloroform/methanol (2:1, v/v) (22) containing 453 nmol/L BHT. The extracts were dried under N_2 and the residue was dissolved in 1 mL of toluene (guaranteed reagent; Nacalai tesque) and applied to a Sep-Pak Vac silica cartridge (Waters Japan) to separate oxysterols from sterols (23). The cartridge was sequentially eluted with 1 mL of hexane, 8 mL of 2-propanol (5 mL 2-propanol/L hexane), and 5 mL of 2-propanol (300 mL 2-propanol/L hexane), which allowed for the sequential elution of 5α-cholestane, cholesterol plus phytosterol, and 19-hydroxycholesterol plus oxysterols, respectively. The recovery of individual oxycholesterols and oxyphytosterols from the cartridge was confirmed to be almost 100%. The samples were allowed to saponify at room temperature overnight

TABLE 1

Composition of oxyphytosterols in Studies 1 and 2[1]

	Study 1	Study 2
	g/kg	
Oxycampesterols		
7α-Hydroxycampesterol	58 ± 5	10 ± 0
7β-Hydroxycampesterol	58 ± 3	28 ± 3
β-Epoxycampesterol	51 ± 4	62 ± 1
α-Epoxycampesterol	74 ± 6	76 ± 2
Dihydroxycampesterol	40 ± 4	18 ± 0
7-Ketocampesterol	40 ± 6	125 ± 3
Oxysitosterols		
7α-Hydroxysitosterol	85 ± 8	16 ± 1
7β-Hydroxysitosterol	78 ± 4	33 ± 1
β-Epoxycampesterol	57 ± 7	69 ± 1
α-Epoxycampesterol	104 ± 8	53 ± 1
Dihydroxycampesterol	54 ± 5	15 ± 1
7-Ketocampesterol	57 ± 8	130 ± 1
Unknown	243 ± 72	316 ± 11
Campesterol	0	23 ± 1
β-Sitosterol	0	27 ± 0

1 Values are means ± SEM of triplicate analyses.

in the dark (24), and unsaponified lipids were converted into trimethylsilylethers in a mixture of trimethylchlorosilane, 1,1,1,3,3,3-hexamethyldisilazane, and dried pyridine (1:3:9, v:v:v) for 30 min at room temperature, as previously described (17,21). GC of sterols was performed as described (25). GC-MS was performed on a Shimadzu GC-17A ver. 3 coupled with the SPB-1 fused silica capillary column connected to a Shimadzu QP5050A series mass-selective detector. The following variables—ions monitored, relative retention time, correlation coefficient for calibration curves, response factors for the monitored ions, detection limit, and the CV for repeated injection—were determined as previously described (17,21).

Determination of 8-iso-PGF2α. Purification and measurement of urinary 8-iso-PGF2α were carried out by combining 4 methods (26–29). The 8-iso-PGF2α was analyzed by GC negative ion chemical ionization mass spectrometry (GC/NICI-MS). GC/NICI-MS was performed using a Shimadzu QP5050A GC/MS (Shimadzu). GC was performed using a 30-m, 0.25-mm-diameter, 0.25-μm-film-thick, SPB-20 fused silica capillary column (Supelco). The column temperature was initially maintained at 100°C for 2 min. The column was then heated to 250°C in 7 min and then to 290°C at 2°C/min and maintained at this temperature (29). Isobutane was used as a reactant gas for negative chemical ionization and helium as a carrier gas at 1.1 mL/min. The ion source temperature was 290°C and the electron energy was 70 ev. The ion monitor for endogenous 8-iso-PGF2α was set at m/z 569 (M-181). We used 8-iso-PGF2α-d4 (Cayman Chemicals) as an internal standard and ions at m/z 573 were monitored. Quantification of endogenous 8-iso-PGF2α was accomplished by selected ion monitoring analysis of the ratio of m/z 569 to m/z 573. The lower limit of detection (signal-to-noise ratio of 4:1) of 8-isoPGF2α was within the range for 50 μL urine. A standard curve was constructed by adding varying amounts of unlabeled 8-iso-PGF2α to 1 ng of 8-isoPGF2α-d4, and the measured ratio of m/z 569 to m/z 573 and the expected ratio were compared. The standard curve was linear over a 30-fold range in concentration. A concentration of urine 8-iso-PGF2α was expressed as a function of urinary creatine (Wako Pure Chemicals).

Analyses of serum and lymph lipids. Serum lipid levels were measured using commercially available kits (cholesterol C test, triglyceride G test, and phospholipid B test, Wako Pure Chemicals). Lymph lipids were chemically determined as previously described (21).

Morphometric determination of atherosclerosis. Apo E–deficient mice were perfused with 50 mL PBS (pH 7.4) via a cannula inserted into the left ventricle, which allowed unrestricted efflux from an incision in the vena cava. After the aorta and its main branches were dissected from the aortic valve to the iliac bifurcation, perfusion of the heart was immediately continued with 50 mL 10% (v/v) neutral formalin buffer solution (pH 7.4). The heart was removed and fixed in 10% (v/v) neutral formalin buffered solution (19). Hearts containing aortic roots were processed for quantitative atherosclerosis assay to determine the cross-sectional lesion volume as previously described (17,30).

Statistics. Results are expressed as means ± SE. Statistical analysis was carried out with Statcel (Excell 2000). A paired t test was used for comparisons within groups. Student's t test was used to compare 2 groups after Bartlett's test was used to check that variances were homogenous. Statistical comparison of 3 or more groups was done by Fisher's PLSD method, following detection of an effect by one-way ANOVA. Differences were considered significant at $P < 0.05$.

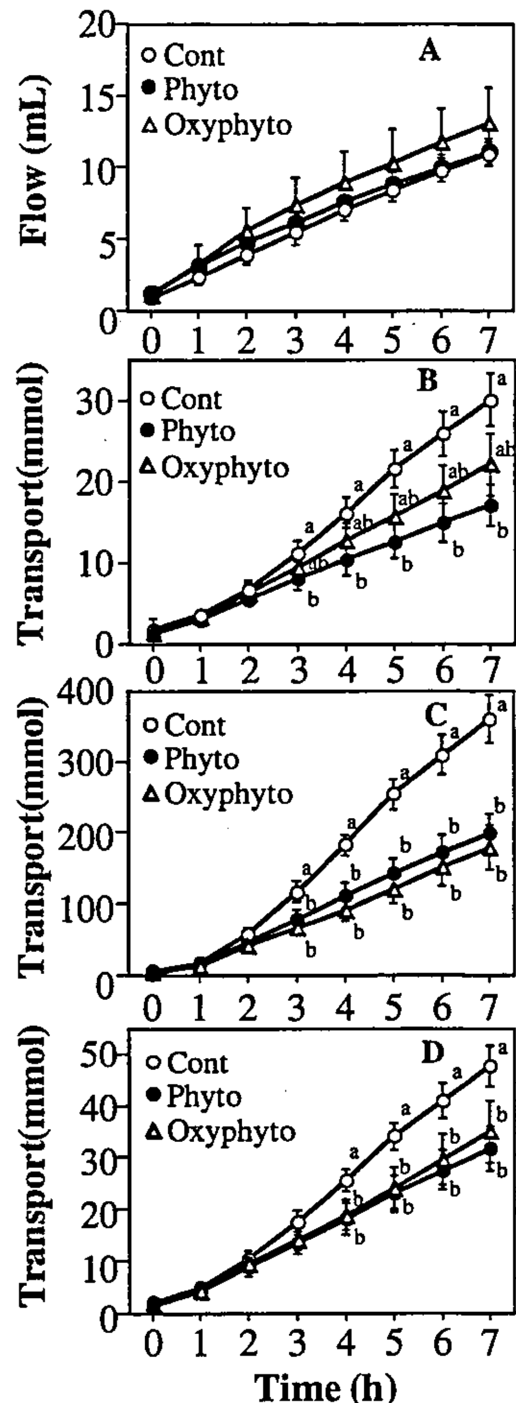

FIGURE 1 Lymph flow (*A*) and the cumulative transport of cholesterol (*B*), triacylglycerols (*C*), and phospholipids (*D*) in rats fed a control (Cont, n = 7), phytosterol (Phyto, n = 12), or oxyphytosterol (Oxyphyto, n = 10) diet. Values are means ± SEM. Means without a common letter differ, $P < 0.05$.

RESULTS

Study 1. Food consumption by the rats within 30 min did not differ among the groups (7.43 ± 0.57 g, n = 7; 6.43 ± 0.49 g, n = 12; 7.70 ± 0.33 g, n = 10, for the control, phytosterol, and oxyphytosterol groups, respectively). Lymph flow also did not differ among the groups (**Fig. 1**). Rats fed the phytosterol diet transported less cholesterol, triacylglycerols, and phospholipids for 3–7 h after the meal than controls. Rats fed the oxyphytosterol diet also transported less triacylglycerols and phospholipds than did control diet-fed rats. Rats fed the oxyphytosterol diet transported less cholesterol at 5 h after the meal than controls. Lipid transport did not differ between the phytosterol- and oxyphytosterol-fed groups.

In the basal lymph collected prior to the consumption of oxyphytosterols, the following oxidized campesterols and sitosterols were detected (in nmol/h, n = 10): 7α-hydroxycampesterol (1.36 ± 0.60), 7β-hydroxycampesterol (0.81 ± 0.24), β-epoxycampesterol (0.50 ± 0.19), α-epoxycampesterol (0.74

± 0.31), dihydroxycampesterol (0.69 ± 0.28), 7-ketocampesterol (0.70 ± 0.27), 7α-hydroxysitosterol (2.07 ± 1.02), 7β-hydroxysitosterol (1.15 ± 0.50), β-epoxysitosterol (0.49 ± 0.16), α-epoxysitosterol (0.79 ± 0.34), dihydroxysitosterol (0.83 ± 0.28), and 7-ketositosterol (1.10 ± 0.60). After the meal, the lymphatic recovery of oxycampesterols was greater than that of the corresponding oxysitosterols, except for the dihydroxy oxysterols (Fig. 2A). Among the oxycampesterols, the lymphatic recoveries of 7β- and 7α-hydroxy were greater than those of the other oxyphytosterols, as was also the case for oxysitosterols. The lymphatic recoveries (on a mass basis) of the 6 oxycampesterols (15.9 ± 2.8%, n = 10) and 6 oxysitosterols (9.12 ± 1.77%, n = 10) were greater than those of the corresponding unoxidized campesterol (5.47 ± 1.02%, n = 12) or β-sitosterol (2.16 ± 0.37%, n = 12) (Fig. 2A, B). Among the lymphatic sterols, the recovery (on a mass basis) of campesterol was greater than that of β-sitosterol or stigmasterol (1.39 ± 0.33%, n = 12), but lower than for cholesterol (37.3 ± 8.3%, n = 6) (Fig. 2B).

Study 2. The phytosterol and oxyphytosterol diets did not affect the body weight gain, food intake, or liver weight of apo E–deficient mice (results not shown). Serum cholesterol concentrations did not differ between mice fed phytosterol (13.7 ± 1.1 mmol/L) and mice fed oxyphytosterol (16.0 ± 1.9 mmol/L) diets. The liver cholesterol concentration in the mice fed the oxyphytosterol diet (12.7 ± 0.5 mmol/kg, n = 7) was greater than that in phytosterol diet–fed mice (14.9 ± 0.9 mmol/kg, n = 7) ($P < 0.05$).

Even in mice fed the phytosterol diet, there was a small but measurable amount of oxyphytosterols in the serum, liver, and aorta (Table 2). Mice fed oxyphytosterols had significantly more oxyphytosterols in these tissues than mice fed phytosterols. However, the concentrations of oxysitosterols in the aorta

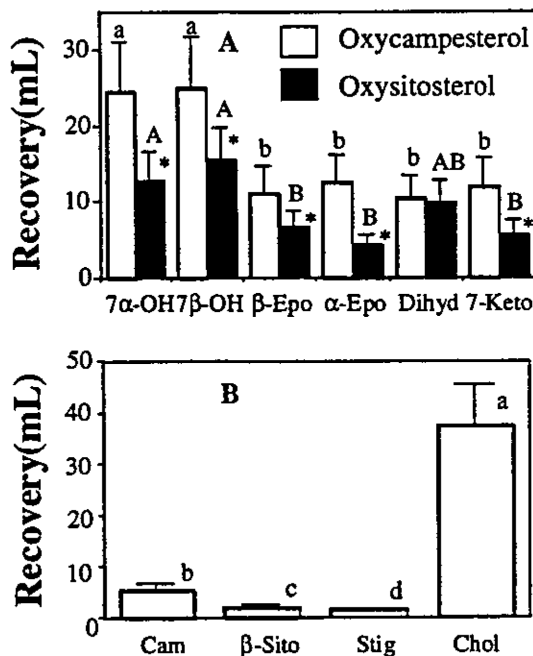

FIGURE 2 Lymphatic recovery of oxyphytosterols (*A*) in rats fed an oxyphytosterol (n = 10) and sterols (*B*) in rats fed a phytosterol (n = 12) or control (n = 7) diet. Values are means ± SEM. Means without a common letter differ, $P < 0.05$. *Different from the corresponding oxycampesterols, $P < 0.05$. Abbreviations: β-Epo, β-epoxy; α-Epo, α-epoxy; β-Sito, β-sitosterol; Cam, campesterol; Chol, cholesterol; Dihyd, dihydro; Stig, stigmasterol

did not differ between the 2 groups, except that the oxysterol fed–mice had a higher concentration of 7β-hydroxysitosterol than the phytosterol fed–mice. In mice fed the oxyphytosterol diet, 7β-hydroxycampesterol and 7β-hydroxysitosterol were the most abundant species of oxyphytosterol in the serum, liver, and aorta, although they were not the most abundant species in the diet (Table 1). Mice fed phytosterols had significantly more campesterol and β-sitosterol in the serum, liver, and aorta than mice fed oxyphytosterols. The concentrations of campesterol in these tissues were higher than that of β-sitosterol ($P < 0.05$). Unlike phytosterols, the concentrations of total oxycampesterols in the serum and liver of the 2 groups were almost the same as that of the total oxysitosterols.

Mice fed oxyphytosterols had significantly higher serum concentrations of 7α-hydroxycholesterol, 7β-hydroxycholesterol, dihydroxycholesterol, 7-ketocholesterol, and 25-hydroxycholesterol in the serum than the phytosterol-fed mice (Table 3). In particular, the elevation of 25-hydroxycholesterol (~600%) was greater than that of other oxycholesterols. However, serum concentrations of α-epoxycholesterol, β-epoxycholesterol, and 27-hydroxycholesterol did not differ between the groups.

Cholesterol concentrations in the aorta and the lesion area in the aortic root did not differ between the phytosterol and oxyphytosterol groups (data not shown). Furthermore, dietary oxyphytosterol did not affect the amount of urinary 8-iso-PGF2α excreted (data not shown).

DISCUSSION

The present study on thoracic duct lymph from rats confirmed the discriminatory capacity of the small intestine between cholesterol and plant sterols, in that cholesterol is preferentially absorbed and transported into the lymph over plant sterols (31–33). The pattern of discrimination depends on the side chain; the addition of 1 methyl group at the C-24 position (campesterol), 1 ethyl group at the C-24 position (β-sitisterol), or 1 ethyl group at the C-24 position, and the Δ22 double bond (stigma sterol). Because the uptake of cholesterol and plant sterols from the intestinal lumen into enterocytes is a rapid process in mice (34), the efflux rather than uptake of sterols may be the main process that discriminates between the lymphatic transport of sterols (35,36). Cholestery ester synthesis in enterocytes via acyl-coenzyme A:cholesterol acyltransferase (ACAT) might be a crucial step for discrimination in that cholesterol is preferentially esterified. Therefore, less free cholesterol than free plant sterols is available for transport out of the cells and back into the intestinal lumen (25,33). β-Sitosterol is a poorer ACAT1 substrate than cholesterol in mixed micelles and reconstituted vesicles (37).

For the lymphatic absorption of oxidation products of campesterol and β-sitosterol, the pattern of discrimination depended on the side chain: lymphatic recovery of oxycampesterols was higher than that of oxysitosterols, except for their trihydroxy types. Of note, the lymphatic recovery of oxyphytosterols was higher than that of unoxidized plant sterols. In particular, the addition of a hydroxyl group (7α-OH and 7β-OH) to the 7 position of campesterol and β-sitosterol resulted in the greatest recovery in the lymph. These results contradicted our previous results for cholesterol with a second oxygen atom, present as a carbonyl, hydroxyl, or epoxide group in the cyclopentanoperhydrophenanthrene nucleus, in that it was transported at a reduced rate into the lymph compared with unoxidized cholesterol (21). In that study, recoveries of α-epoxycholesterol in the lymph were the highest. Further-

TABLE 2

Oxyphytosterol and phytosterol levels in serum, liver, and aorta of apo E–deficient mice fed a phytosterol or oxyphytosterol diet[1]

	Serum		Liver		Aorta	
Sterols	Phytosterol[2]	Oxyphytosterol[3]	Phytosterol	Oxyphytosterol	Phytosterol	Oxyphytosterol
	μmol/L		*nmol/g*			
Plant sterols						
Campesterol	354.4 ± 42.4	80.1 ± 42.2*	227.9 ± 14.7	51.7 ± 3.2*	117.5 ± 19.2	25.7 ± 2.0*
β-Sitosterol	43.4 ± 5.1	14.2 ± 5.1*	21.6 ± 1.3	8.1 ± 0.7*	48.0 ± 10.4	16.9 ± 1.4*
Oxidized campesterols						
7α-Hydroxy	0.08 ± 0.01	1.50 ± 0.01*	0.07 ± 0.00	0.55 ± 0.14*	0.31 ± 0.05	0.31 ± 0.07
7β-Hydroxy	0.48 ± 0.05	23.04 ± 0.05*	0.14 ± 0.00	13.20 ± 2.04*	1.15 ± 0.38	6.05 ± 1.06*
β-Epoxy	0.28 ± 0.04	4.03 ± 0.04*	0.22 ± 0.05	1.75 ± 0.41*	0.31 ± 0.05	0.94 ± 0.14*
α-Epoxy	0.32 ± 0.06	6.07 ± 0.06*	0.24 ± 0.05	4.70 ± 0.53*	0.46 ± 0.07	1.42 ± 0.34*
Dihydroxy	0.81 ± 0.05	2.42 ± 0.05*	0.87 ± 0.14	2.09 ± 0.21*	0.83 ± 0.14	1.06 ± 0.23
7-Keto	0.16 ± 0.02	7.02 ± 0.02*	0.19 ± 0.02	2.44 ± 0.65*	0.51 ± 0.10	1.01 ± 0.15*
Total	2.14 ± 0.18	43.85 ± 0.18*	1.77 ± 0.17	24.56 ± 3.58*	3.51 ± 0.38	10.75 ± 1.86*
Oxidized β-sitosterols						
7α-Hydroxy	0.21 ± 0.03	1.87 ± 0.03*	0.14 ± 0.02	0.70 ± 0.14*	3.07 ± 0.19	2.51 ± 0.58
7β-Hydroxy	0.68 ± 0.07	24.29 ± 0.07*	0.23 ± 0.02	13.30 ± 1.81*	1.32 ± 0.30	3.99 ± 0.93*
β-Epoxy	0.19 ± 0.02	3.04 ± 0.02*	0.37 ± 0.09	1.56 ± 0.37*	0.95 ± 0.14	1.81 ± 0.44
α-Epoxy	0.09 ± 0.02	3.37 ± 0.02*	0.21 ± 0.07	3.30 ± 0.37*	0.77 ± 0.23	1.11 ± 0.12
Dihydroxy	0.28 ± 0.04	1.41 ± 0.04*	0.11 ± 0.02	1.18 ± 0.18*	2.23 ± 0.51	2.39 ± 0.40
7-Keto	0.34 ± 0.08	1.89 ± 0.08*	0.54 ± 0.09	1.21 ± 0.19*	1.72 ± 0.33	1.42 ± 0.28
Total	1.78 ± 0.21	35.72 ± 0.21*	1.57 ± 0.21	21.18 ± 2.42*	10.06 ± 0.72	13.25 ± 1.92

[1] Values are means ± SEM, $n = 7$; * different from the phytosterol group, $P < 0.05$.
[2] The phytosterol diet contained 0.2 g/kg phytosterol mixture.
[3] The oxyphytosterol diet contained 0.2 g/kg heat-prepared oxyphytosterols.

more, the present study also revealed that the addition of a second oxygen atom to the cyclopentanoperhydrophenanthrene nucleus of phytosterols lowered their ability to reduce the absorption of cholesterol into the lymph (Fig. 1). These data suggest that the intestine has a complex pattern for discriminating between the cyclopentanoperhydrophenanthrene nucleus and the side chain of sterols for their absorption and lymphatic transport.

In contrast to our observations, Grandgirad et al. (38) reported that lymphatic recoveries of oxyphytosterols (7-keto and epoxides) in rats are lower than that of β-sitosterol and

TABLE 3

Oxycholesterol levels of serum in apo E–deficient mice fed a phytosterol or oxyphytosterol diet[1]

Sterols	Phytosterol[2]	Oxyphytosterol[3]
	μmol/L	
7α-Hydroxy	0.454 ± 0.020	0.787 ± 0.107*
7β-Hydroxy	0.323 ± 0.010	1.110 ± 0.171*
β-Epoxy	3.775 ± 0.695	3.154 ± 0.720
α-Epoxy	4.246 ± 0.596	5.215 ± 0.571
Dihydroxy	0.047 ± 0.007	0.074 ± 0.006*
7-Keto	0.107 ± 0.010	0.252 ± 0.022*
25-Hydroxy	0.042 ± 0.004	0.298 ± 0.040*
27-Hydroxy	0.273 ± 0.025	0.315 ± 0.032
Total	9.267 ± 0.469	11.205 ± 1.480

[1] Values are means ± SEM, $n = 7$; * different from the phytosterol group, $P < 0.05$.
[2] The phytosterol diet contained 0.2 g/kg phytosterol mixture.
[3] The oxyphytosterol diet contained 0.2 g/kg heat-prepared oxyphytosterols.

campesterol, respectively. However, their results in which oxycampesterols were better absorbed than oxysitosterols are in agreement with those of the present study. The discrepancies between the 2 studies might be due to the different methods used to administer oxyphytosterols, collect lymph, and analyze oxyphytosterols. They administered oxyphytosterols into the stomach of thoracic lymph duct cannulated rats, collected the lymph immediately after the operation, and analyzed sterols with GLC.

Reflecting the lymphatic transport of sterols in rats, apo E–deficient mice fed a diet of oxyphytosterols had elevated amounts of these compounds in the serum, liver, and aorta compared to mice fed the phytosterol diet. The concentrations of 7β-hydroxycampesterol and 7β-hydroxysitosterol in the serum, liver, and aorta were higher than that of other oxyphytosterols. Furthermore, the concentration of campesterol in the serum, liver, and aorta was consistently higher than the concentration of β-sitosterol. 7β-Hydroxysitosterol and 7β-hydroxycampesterol are present in the serum of phytosterolemic patients, who have highly elevated serum plant sterol concentrations (10). These results might be due to discrimination by the circulatory system for 7β-hydroxysterols and other sterols and between campesterol and β-sitosterol. Some investigators hypothesized that ABC monomers (ABCG5, ABCG8) expressed in the liver and intestine (39) are able to discriminate between cholesterol and other sterols (40,41). Therefore, the circulatory system appears to have a discrimination system that eliminates campesterol and 7β-hydroxyphytosterols less efficiently compared with β-sitosterol and other oxyphytosterols.

In the present study, apo E–deficient mice fed a diet containing oxyphytosterols had elevated levels of 5 kinds of oxycholesterols (7α-hydroxycholesterol, 7β-hydroxycholesterol, dihydroxycholesterol, 7-ketocholesterol, 25-hydroxycholesterol) in the serum compared to mice fed the phytosterol diet.

The groups did not differ in the concentrations of β-epoxycholesterol, α-epoxycholesterol, and 27-hydroxycholesterol. Although a portion might be formed during the extraction and analyses, oxysterols formed in vivo include 7α-hydroxycholesterol, 7β-hydroxycholesterol, 7-ketocholesterol, and 24-, 25-, and 27-hydroxycholesterols, as shown by $^{18}O_2$ inhalation [reviewed by Björkhem (42)]. Because serum cholesterol did not differ between the groups, it is likely that elevated oxycholesterol in mice fed the oxyphytosterol-containing diet was due to the consumption of oxyphytosterols. Rosenblat and Aviram (43) showed that the enrichment of peritoneal macrophages from C57BL6 mice with 3 kinds of oxysterols (7-ketocholesterol, β-epoxycholesterol, and 7β-hydroxycholesterol) dose-dependently increased in superoxide anion release and cell-mediated oxidation of LDL.

In the present study, the adverse effects of oxyphytosterols on the development of atherosclerosis were evaluated in apo E–deficient mice by measuring aortic cholesterol levels and lesion size in the aortic root. Oxidative stress was also measured through the urinary excretion of 8-iso-PGF2α, one of the most reliable biomarkers of oxidative stress in vivo (44). Although comparisons to mice fed the AIN diet alone were not performed, our results showed for the first time that there was no significant difference in the size of lesions or aortic cholesterol concentration in apo E–deficient mice fed the phytosterol- and oxyphytosterol-supplemented diets. These results agree with our previous results, which showed no significant effect of dietary oxycholesterols on the development of atherosclerosis in apo E–deficient mice (17). Furthermore, the oxyphytosterol and phytosterol groups did not differ in creatine-indexed urinary isoprostanes. Accordingly, although apo E–deficient mice have a high antibody titer against oxidized lipids (45) and dietary oxyphytosterols might locally accelerate oxidative stress as reflected by the elevation of oxycholesterol in the serum, the endogenous antioxidant defense system and dietary antioxidants are apparently adequate to minimize the amounts of in vivo oxidant damage (5,17). Alternatively, some dietary oxysterols might exert a beneficial effect on the development of atherosclerosis. Oxycholesterols such as 22(R)-hydroxycholesterol, 20(S)-hydroxycholesterol, 24-hydroxycholesterol, 25-hydroxycholesterol, 7α-hydroxycholesterol, and 27-hydroxycholesterol are ligands for the liver X receptor (LXR). This receptor is a transcription factor for the ATP-binding cassette transporter A1, which is involved in cholesterol efflux from the arterial intima (46). Similarly, oxyphytosterols derived from ergosterol and brassicasterol are LXR agonists, and act as effectively as the ligands 22(R)-hydroxycholesterol and 24(S), 25-epoxycholesterol (47). Therefore, oxycholesterol and oxyphytosterol may exert their effects on the sterol regulatory machinery and thereby prevent the development of atherosclerosis. This hypothesis, however, will need to be tested in future studies.

In summary, the present study showed that the intestine and liver possess a discrimination system that eliminates less campesterol and 7β-hydroxyphytosterols. Dietary oxyphytosterols are absorbed and accumulate in the serum, liver, and aorta in apo E–deficient mice. However, dietary oxyphytosterols do not affect aortic cholesterol or the lesion volume in the aortic valve compared to phytosterols. The results indicate that exogenous oxysterols do not promote the develomment of atherosclerosis in apo E–deficient mice.

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Mechanisms of Phytosterolemia in Stroke-Prone Spontaneously Hypertensive and WKY Rats

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Analysis of sterol composition in serum, liver, adipose tissue, adrenals, and abdominal aorta demonstrated that the contents of plant sterols, campesterol and sitosterol, were evidently higher in WKY and stroke-prone spontaneously hypertensive (SHRSP) rats than in Wistar and WKA rats fed a diet containing a 0.5% plant sterol mixture. Lymphatic 24-hour recovery of ^{3}H-sitosterol was about 2-fold higher in the WKY and SHRSP rats than in the WKA rats. Lymphatic absorption of ^{14}C-cholesterol was also higher in WKY and SHRSP rats compared with WKA rats, but the difference was smaller than in the case of sitosterol. The remarkable increase of sitosterol absorption in WKY and SHRSP rats was observed between 9 and 24 hours after the administration. In SHRSP rats, lymphatic absorption of sitosterol between 0 and 3 hours was also higher than those in the other rat strains. Markedly less esterified ^{3}H-sitosterol was detected in lymph than ^{14}C-cholesterol in all strains, and in WKY and SHRSP rats, only a small increase in the esterified forms of sitosterol and cholesterol was observed. Although the incorporation of micellar ^{3}H-sitosterol and ^{14}C-cholesterol into intestinal brush border membranes was higher in SHRSP rats than in WKA rats, no difference was observed between WKY and WKA rats. These observations suggest that the incorporation into the brush border membranes and the esterification of sterols are not the major determinants for the hyperabsorption of sitosterol and cholesterol in SHRSP and WKY rats. Secretion of sitosterol and cholesterol in the bile of rats fed a plant sterol mixture was lower in SHRSP than in WKA rats. These results suggest that WKY and SHRSP strains deposit plant sterols in the body by enhancing the absorption and lowering the excretion of plant sterols. These strains of rats may be suitable models for studying mechanisms of differential absorption of various sterols.
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IN ALMOST ALL animal models and humans, intestinal absorption of plant sterols is undoubtedly low compared with cholesterol.[1,2] The mechanism underlying the discrimination of absorption of these sterols is an enigma yet to be solved.[3] Our previous study suggested a possibility that the low absorbability of sitosterol may be due to the high affinity for bile salt micelles and hence, a release of sitosterol as a monomer from the micelles and the incorporation into intestinal brush border membranes are less efficient.[3] However, it is known that phytosterolemic patients who deposit plant sterols in their body absorb plant sterols more efficiently than normal subjects.[4,5] Because phytosterolemia is a rare inherited disease, it is assumed that carrier proteins are involved in the hyperabsorption of plant sterols. Although gene defects in 10 families with phytosterolemia were localized to chromosome 2p21,[6] no candidate genes were found in this region and hence, mechanisms underlying the high absorbability of plant sterols in these patients have not yet been elucidated. The reason for this uncertainty is attributed to a lack of an appropriate animal model for this issue. We report here that the 3 strains of WKY, spontaneously hypertensive (SHR) and stroke-prone spontaneously hypertensive (SHRSP) rats, can deposit plant sterols to a significant extent as compared with Wistar and WKA rats. The SHRSP strain was genetically separated from the SHR strain,[7] which was originated from the WKY strain.[8] To elucidate causes underlying the deposition of plant sterols in these rat strains, the lymphatic absorption and the incorporation into intestinal brush border membranes of plant sterols were examined. It has been reported that the secretion of plant sterols in bile in sitosterolemic patients is less efficient than that in normal subjects.[4] This may be another reason for the high deposition of plant sterols in their body. Therefore, biliary excretion of sterols was also measured in SHRSP and WKA rats.

MATERIALS AND METHODS

Materials

[22,23(n)-^{3}H] Sitosterol (specific activity 814 GBq/mmol, Amersham, Buckinghamshire, England) was kindly provided from Kao Co, Tokyo, Japan; [4-^{14}C]cholesterol (55 mCi/mmol) was purchased from Amersham.

Feeding Study of Plant Sterols

Groups of 9-week-old male WKY (WKY/NCrj, inbred, SPF, Charles River Japan, Kanagawa, Japan), SHRSP (SHRSP/Sea, inbred, SPF, Seac Yoshitomi, Fukuoka, Japan), Wistar (Sea: Wistar, outbred, SPF, Seac Yoshitomi) and WKA (WKA/Sea, inbred, SPF, Seac Yoshitomi) rats were fed a purified diet (AIN-93G)[9] containing 10% safflower oil and 0.5% plant sterol mixture (Merck, Darmstadt, Germany) for 2 weeks. The composition of the sterol preparation was in weight percent, sitosterol, 54.9; stigmasterol, 3.9; campesterol, 37.6; campestanol, 0.4; and sitostanol, 1.5. The chemical structure of some plant sterols is shown in Fig 1. At the termination of the feeding period, rats were fasted for 7 hours, and blood was then withdrawn from the abdominal

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