チンと糖尿病の関連が示唆されており，糖尿病患者の血中ビオチン濃度が健常人と比較して低いことや，ビオチン投与によって糖尿病の症状が改善することが報告されている[11)12)13)]．また，ヒストンのビオチン化が細胞増殖においてDNA合成に大きな役割を果たしていることが明らかにされており，Jurkat細胞では，ビオチン欠乏状態にしてもヒストンのビオチン化が正常に保たれることが報告されている[14)]．

鳥類は，ビタミン要求性が高く，ビオチンに関しても例外ではない[15)]．鳥類の卵黄中には，ビオチンが多量に含まれている．食餌から摂取されたビオチンの多くが卵に移行し，卵黄にはビオチンが多量に蓄積している[16)]．このことから，ビオチンが鳥類の胚発育に重要な役割を担っていると考えられる．そこで，本研究では，ニワトリを用いて，母体から卵胞あるいは卵黄へビオチンが供給される機序を，卵胞の発達段階によって分析し，卵形成におけるビオチンの栄養学的意義を検討した．

実験材料および実験方法

1．実験材料

ニワトリの卵胞および卵黄（いずれも無精卵）は，産卵用の白色レグホン5羽から採取した（Table 1）．ニワトリは屠殺前に採血し，遠心分離後，血清を得た．また，屠殺後，直ちに卵巣，卵管，肝臓を摘出した．

ニワトリの卵巣は，肝臓前方に位置しており，卵巣間膜によって吊るされ，白色や黄色の球形をした大小様々な卵胞が，各発達段階によってブドウ状に集合している[17)18)19)20)21)]．卵巣内の卵細胞の数は肉眼で数えられるもので約1,900個，顕微鏡下で確認できるもので12,000個以上存在している．これらの卵胞は，成長して卵黄として排卵されるまでに数年を要する．直径1mm以下の卵胞が5mm程度に成長するには数ヶ月以上，さらに約10mmに成長するに数ヶ月を要する．その後，卵胞は排卵直前の10日前後で急激に数cmまで成長する．また，ニワトリでは約27時間の間隔で排卵がおこなわれ，排卵された卵胞は，全長70cm程度の卵管を，約24時間かけて移動することが知られている．ニワトリの卵管は，上部から漏斗部，膨大（卵白分泌）部，峡部，卵管腺（子宮）部および膣部に分類され，排卵された卵胞は，卵管を移動する間に，卵白，卵殻膜，卵殻の形成の段階を経て，完全な卵として体外へ放出させる機能を持っている．

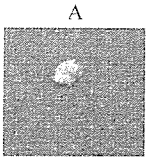
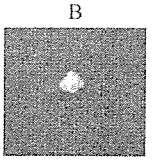
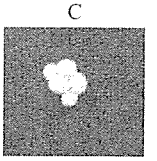
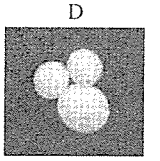
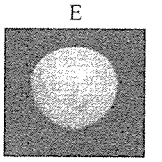
これらのことから，本研究では，卵胞の成長の過程により，大きさから，便宜的に5段階のステージに分類した（Table 1）．具体的に，単独で採取することが不可能な1mm以下の卵胞をステージA（発生初期），肉眼で数えることができ，個体での採取が可能な1mm以上5mm未満のものをステージB（成長前期），5mm以上10mm未満のものをステージC（成長後期），排卵が近づき急成長した段階で10mm以上のものをステージD，卵巣内で最も大きく成長した排卵直前のものをステージEとした．卵胞は，ニワトリの個体別にステージごとにまとめて1サンプルとした．また，排卵されて卵管内に存在する成熟卵（卵黄のみ）も採取した．排卵後の卵黄は，卵白を完全に取り除くために1/15Mリン酸緩衝液（pH7.2）で洗浄した．このほか，卵管組織を卵管の上部および下部から採取した．卵管上部としては，卵白タンパク質の合成や分泌がおこなわれる膨大部を採取し，下部としてはカルシウムの分泌と卵殻の形成がおこなわれる子宮部を採取した．

血清はそのまま測定試料とし，それ以外の試料に関しては，1/15Mリン酸緩衝液（pH7.2）およびサンプルの0.01%量の界面活性剤（TRITON X-100）を加えて十分にホモジナイズしたものを測定試料とした．また，サンプルはすべて，測定時まで−40℃で凍結保存した．

2．ビオチンの分析

ビオチンの定量は，ビオチン要求株である乳酸菌（*Lactobacillus plantarum* ATCC 8014）を用いた微生物学的

Table 1. Developmental stages of ovarian follicle in laying hens.

Stage	A	B	C	D	E	Egg yolk
						
Size (mm)	＜1	＜5	＜10	＜20	≧20	—
No. of ovarian follicles collected.	—	53	25	9	5	5
Weight (g)	—	＜0.02 (5)	0.16 ± 0.04 (5)	0.68 ± 0.35 (5)	10.5 ± 1.4 (5)	18.5 ± 3.6 (5)

[a]mean ± SD
( ) no. of ovarian follicles analyzed.

定量法[22)23)24)]に従い，比濁測定した．乳酸菌は16時間培養後洗浄し，菌濃度を濁度(OD_{610} = 0.15)で調製したものを接種菌液とした．定量には，ビオチン定量用基礎培地(日水製薬(株)，東京)を用いて18時間培養後，610nmで測定した．

生体試料や食品中に含まれるビオチンは，ほとんどが結合型ビオチンとして存在するため，本研究では総ビオチンと遊離型ビオチンを測定した．結合型ビオチンとは，タンパク質やペプチドと共有結合した状態で存在しているビオチンであり，生体内ではそのまま利用することはできない「貯蔵型」である．一方，遊離型ビオチンとはタンパク質などと結合せず，単独で存在している状態のもので，いわゆる「酵素型」である．生体内でのビオチンの吸収や利用は遊離型ビオチンが関与している．また，総ビオチンに対する遊離型ビオチンの割合を，遊離型ビオチン率(遊離率)とした．ビオチンの測定を概略すると，測定試料に同量の4.5N硫酸を加え，オートクレーブ(121℃，2気圧，60分)で酸加水分解後，4.5N水酸化ナトリウムで中和して測定したものを総ビオチンとした．遊離型ビオチンの定量は，試料を酸加水分解せず，そのままもしくは希釈して測定に供した．なお，ビオチン濃度はng/g(ml)またはμg/gで表した．

3. ビオチニダーゼの活性の測定

ビオチニダーゼには2つの役割がある[25)26)]．1つは，食品や生体内に存在する結合型ビオチンはそのままでは利用できないため，消化管や細胞内でビオチニダーゼによって遊離化される．また，ビオチニダーゼは，ビオチン結合タンパクのひとつとして知られており，ビオチンの吸収，運搬および保持などに関与していることが明らかにされつつある．そこで，ビオチンの動態との関連を検討するため，ビオチニダーゼ活性を測定した．

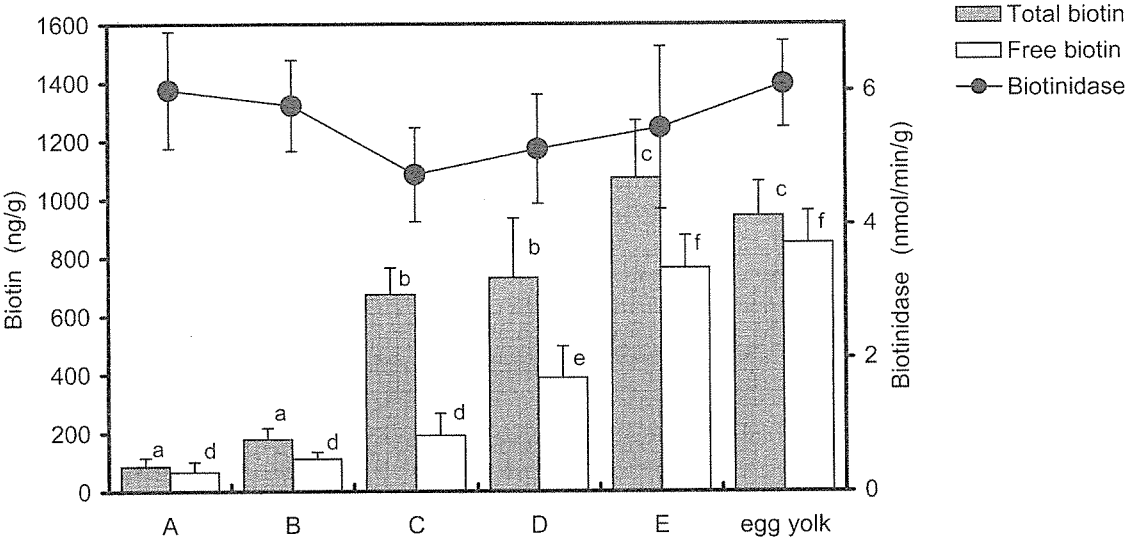
ビオチニダーゼ活性の測定は，Wolfら[27)]の方法に従い，比色測定した．また，活性は，nmol/min/g(ml)で表した．

4. 統計学的解析

データは，平均値±標準偏差で示した。統計処理には，統計ソフトStatView Ver.5.5(SAS Institute Inc., Cary)およびExcel統計Statcel(OMS出版，埼玉)を用いた．また，$p < 0.05$を統計学的に有意な差異があると判定した．

結　果

各ステージにおける卵胞の状態は，ステージAおよびBでは，白色に近い黄色をしており，粘性が低い液体状であった．しかし，ステージCから卵黄の色は次第に濃い黄色を帯び始め，ステージD以降のものでは鮮やかな黄色をしており，粘性も高かった．卵胞のビオチン濃度およびビオチニダーゼ活性をFig. 1に示した．各ステージでの総ビオチン濃度の変化をみると，ステージAでは87.5 ± 29.3ng/gであったが，ステージCで674.1 ± 91.4ng/gと急激に増加し，排卵直前のステージEでは1072.5 ± 197.6ng/gとすべてのステージの中で最も高値であった．また，排卵後の卵黄においても，944.2 ± 116.7ng/gと高値を示した．ビオチン遊離率の変化をみる



	A	B	C	D	E	egg yolk
Percentage of free biotin(%)	75.5±21.1^{g}	64.1±13.0^{gi}	28.2±8.8^{h}	53.4±8.6^{i}	73.9±19.2^{g}	90.7±11.8^{gj}

Fig. 1 Biotin concentrations of ovarian follicle and egg yolk in laying hens.
means ± SD, n = 5
a-b-c, d-e-f, g-h-i-j The data were analyzed by Tukey-Kramer after one-way ANOVA ($p < 0.05$). Means without a common letter differ ($p<0.05$).

と，ステージ A では 75.5 ± 21.1% と高値であったが，次第に減少し，総ビオチン濃度が増加したステージ C では 28.2 ± 8.8% と低値であった．しかし，ステージ C 以降では，卵胞の成長とともに遊離率は再び高くなり，排卵直前のステージ E では 73.9 ± 19.2%，排卵後の卵黄では 90.7 ± 11.8% であった．一方，卵胞のビオチニダーゼ活性に有意な変化はみられなかった．

ニワトリの血清および肝臓のビオチン濃度を Fig. 2-A に示した．総ビオチン濃度は，血清では 52.0 ± 9.3ng/ml，肝臓では 2.9 ± 0.7 μg/g であり，遊離型ビオチン濃度はそれぞれ 26.2 ± 2.7ng/ml および 1.6 ± 0.1 μg/g であった．また，ビオチニダーゼ活性は，血清で 20.2 ± 3.9nmol/min/ml，肝臓で 19.4 ± 0.3nmol/min/g であった（Table 2）．卵管組織のビオチン濃度を示したのが Fig. 2-B である．総ビオチン濃度は卵管上部の膨大部で 45.6 ± 14.0ng/g，下部の子宮部で 49.0 ± 12.1ng/g であり，遊離型ビオチン濃度はそれぞれ 4.8 ± 1.5ng/g および 3.8 ± 2.5ng/g と低値であった．膨大部と子宮部のビオチニダーゼ活性は，それぞれ 10.1 ± 0.3nmol/min/g および 9.4 ± 0.1mnol/min/g であった．

考　察

ビオチンがほ乳動物の胎児や鳥類の胚の発育に不可欠であることが知られている．ほ乳動物では，妊娠マウス（ICR）に乾燥卵白を 25% 添加した精製飼料を与えると，90% 以上の胎児に口蓋裂，小顎症，短肢症などの外表奇形が認められる[4,5,28]．また，C57BL マウスおよび CD-1 マウスでも，ビオチン欠乏によって口蓋や四肢に異常が観察される．A/Jax マウスは，口蓋裂の自然発症率が高い系統であるが，ビオチン欠乏による口蓋裂誘発率はあまり高くないことが報告されている[5]．ハムスターにおいても，妊娠中のビオチン欠乏によって，胎児発育の抑制や，吸収胚や死亡胎児の増加がみられている[6]．一方，ラットでは妊娠中のビオチン欠乏状態によって，胎児に体重の低下はみられるが，形態異常や組織学的変化は認められていない[5]．このように，ほ乳動物では動物種や系統によって感受性が異なるものの，妊娠中にビオチンが欠乏すると胎児に発育の抑制や形態異常が起こることから，ビオチンは胎児の形態形成や発育に重要な役割を果たしていることが示唆されている．

一方，鳥類のビオチン欠乏状態の生理生殖機能への影響に関しては，古くから報告されている．ニワトリや七面鳥では，ビタミン要求性が高いため，精製飼料を与えることで容易にビオチン欠乏状態に陥ることが知られている[15,29]．若い雌レグホン鶏に，低ビオチンの精製試料を与えると，成長が抑制されたり，皮膚炎などの症状が現れる．また，ビオチン欠乏によって，産卵数には影響

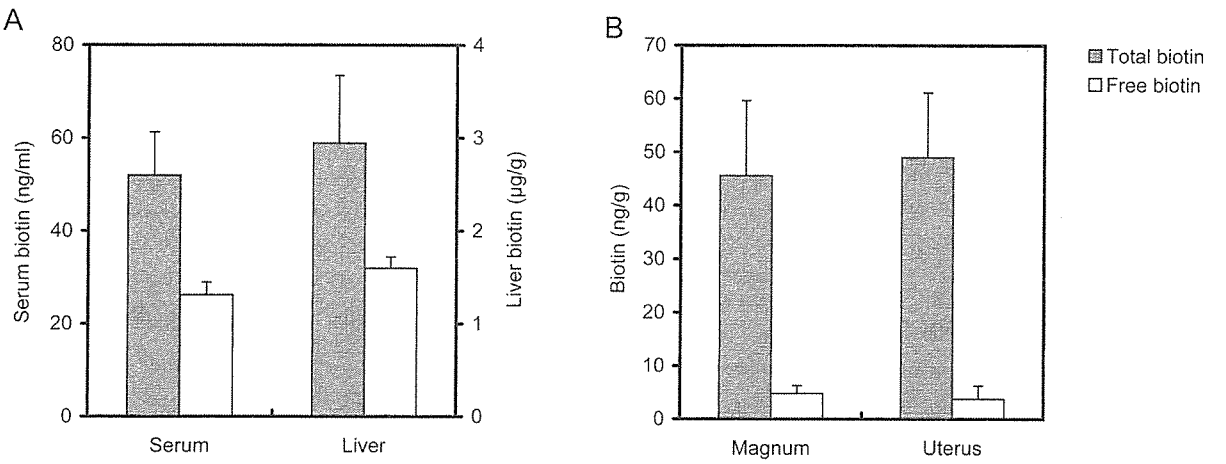


Fig. 2　Biotin concentrations of serum, liver and oviduct in laying hens.
A: serum and liver, B: oviduct
means ± SD, n = 5

Table 2.　Biotinidase activity of serum and organs laying hens.

Biotinidase nmol/min/ml(g)	Serum	Liver	Magnum	Uterus
	20.2 ± 3.9[a]	19.4 ± 0.3	10.1 ± 0.3	9.4 ± 0.1

[a]mean ± SD, n = 5

しないが，胚の死亡率が増したり，孵化率が著しく低下することが知られている[3)30)31)32)]．低ビオチン精製飼料を与えられた親鳥から孵化したヒナには，腱麻痺や運動失調，翼脚の骨格異常などが観察される．このような個体にビオチンを与えると孵化率が急激に回復する．

Cravens ら[30)]の報告では，ビオチン欠乏のヒナに軟骨異栄養症や合指症などの骨格の異常が観察されているが，これらの親鳥には皮膚炎などのビオチン欠乏症状はみられていない．また，ニワトリや七面鳥で，飼料にビオチン 0.55mg/kg を添加した場合にも，産卵数の増加がみられ，胚の死亡率が減少し，孵化率が高くなることが報告されている[33)]．このように，親鳥に比べて，胚ではビオチン欠乏による影響を受けやすいと考えられる．しかし，Whitehead[16)]は，飼料にビオチン 0.06－0.40mg/kg を添加しても，飼料の摂取量，産卵数，鶏卵の大きさ，孵化率に変化がみられなかったと報告している．

雌レグホン鶏では飼料からのビオチン摂取量と，鶏卵のビオチン含量には直線的な関係があることが報告されている[16)31)34)]．飼料からビオチンが多量に摂取されると，卵への移行率は低くなるが，摂取されたビオチンの 50％以上が卵に移行する[16)]．とくに卵黄には多量のビオチンが蓄積しているが，卵黄のビオチン濃度が 50ng/g 以下の場合には胚発育はみられず，正常な胚発育を維持するためには 150ng/ g 以上必要であることが報告されている[35)]．本研究での卵黄ビオチン濃度は 944.2 ± 116.7ng/g であることから，正常な胚発育に十分なビオチンが存在するといえる．これらのことから，鳥類でもビオチンが親鳥の正常な生殖生理機能を維持したり，胚の発育や分化を維持するために不可欠であると考えられる．

卵胞のビオチン濃度の変化をみると，卵胞の発生初期や成長前期と比較して，卵胞の成長後期には総ビオチンは 3 倍以上に増加していた．しかし，排卵を約 10 日後に控えた成長後期では，卵胞の成長過程の中で遊離率は最も低値であった．また，排卵直前期には，卵胞の大きさや色，粘性にも顕著な変化がみられ，ビオチン濃度の増加とともに遊離率も高くなった．さらに，排卵後の卵黄ではビオチンの 90% 以上が遊離型で存在した．これらのことから，卵胞の成長に伴って，胚の発育に必要な他の栄養素と同時にビオチンも卵胞に多量に取り込まれている可能性が考えられる．また，初期の卵胞で遊離率が高いことや，排卵直前に遊離型ビオチン濃度が急激に増加していることから，卵胞へのビオチンの供給は遊離型でおこなわれていると考えられる．なお，成長後期では総ビオチン濃度が著しく増加しているにもかかわらず遊離率は低値であるが，これは卵胞が数ヶ月かけて数 mm から約 10mm に成長する間に，徐々に蓄積されたビオチンが結合型で保存されている可能性が考えられる．

一方，卵胞のビオチニダーゼ活性には，全ステージを通して変化はみられなかった．しかし，卵胞の発生初期から遊離率の低下する成長後期にかけて活性は減少し，排卵期に再び増加する傾向がみられた．そこで全ステージの卵胞と卵黄におけるビオチニダーゼ活性とビオチンの遊離率の関連をみると，両者の間には相関（$r = 0.51$, $p < 0.01$）がみられた（Fig.3）．卵胞へのビオチンの取り込みが遊離型でおこなわれていることとあわせて考察すると，ビオチニダーゼが卵胞においてもビオチンの遊離化としての機能を果たしている可能性が考えられる．

排卵前後の卵胞や卵黄へのビオチンの取り込みのおこなわれる部位や時期が明確ではない．今回の結果では，卵管組織のビオチン濃度は膨大部，子宮部ともに低く，遊離率もそれぞれ 10.5％および 7.7％と低値であった．また，ビオチニダーゼにおいても，卵管組織では卵胞や血清と比較して約半分の活性であった．さらに，卵管へ排卵された卵黄は，膨大部ですぐに卵白で覆われてしまうことからも，卵管にビオチンはほとんど存在しておらず，卵黄中のビオチンは，排卵以前に卵巣で取り込まれていることが考えられる．

ニワトリの血清および肝臓では，ビオチン濃度やビオチニダーゼ活性は，ほ乳動物と比較して高値であった．ほ乳動物では，一般飼料を与えたマウスのビオチン濃度は血清で 5－6ng/ml，肝臓で 1.0－1.2 μg/g であり，ヒトでは健常成人における血清ビオチン濃度は 1.6－3.7ng/ml である．ニワトリではマウスと比較して血清で約 10 倍，肝臓で約 3 倍の高値であった．また，血清および肝臓の両方で，ビオチンの 50％以上が遊離型すなわち活性型で存在しており，これは，鳥類におけるビタミン要求性の高いことの証左であると考えられる．

これらの結果から，卵胞中のビオチンは，卵胞の成長

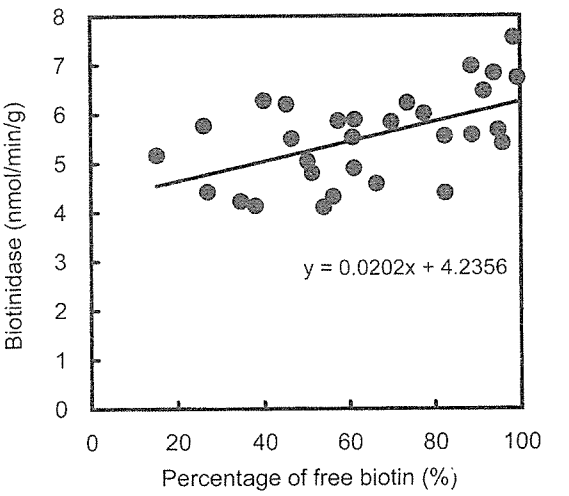


Fig. 3　A linear correlation between biotinidase activity and percentage of free biotin in ovarian follicle.
$r = 0.51$, $p < 0.01$ (Spearman rank correlation)

に伴い，とくに排卵直前に卵巣で多量に取り込まれることが明らかになった．また，卵胞の発生初期から成長期にかけて取り込まれたビオチンは結合型で貯蔵され，排卵直前に再び遊離化されることが示唆された．これは，胚の分化や成長の維持に必要なビオチンが，受精後すぐに利用できる状態にするためと考えられる．以上のことから，ビオチンは鳥類の胚の発育における不可欠な栄養素として，大きな役割を担っていることが示唆された．

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文 献

1) 日本ビタミン学会編（2002）ビタミン研究のブレークスルー，pp231-250，学進出版，大阪
2) Mock DM (2001) Biotin : Handbook of Vitamins. pp397-426, Marcel Dekker., New York
3) Cravens WW, McGibbon WH, Sebesta EE (1944) Effect of biotin deficiency on embryonic development in the domestic fowl. *Anat Rec* **90**, 55-64
4) Watanabe T (1983) Teratogenic effects of biotin deficiency in mice. *J Nutr* **113**, 574-581
5) Watanabe T, Endo A (1989) Species and strain differences in teratogenic effects of biotin deficiency in rodents. *J Nutr* **119**, 255-261
6) Watanabe T (1993) Dietary biotin deficiency affects reproductive function and prenatal development in hamsters. *J Nutr* **123**, 2101-2108.
7) Mock DM, Mock NI, Stewart CW, LaBorde JB, Hansen DK (2003) Marginal biotin deficiency is teratogenic in ICR mice. *J Nutr* **133**, 2519-2525
8) Mock DM, Stadler DD, Stratton SL, Mock NI (1997) Biotin status assessed longitudinally in pregnant women. *J Nutr* **127**, 710-716
9) Zempleni J, Mock DM (2000) Marginal biotin deficiency is teratogenic. *Proc Soc Exp Biol Med* **223**, 14-21
10) Mock DM, Quirk JG, Mock NI (2002) Marginal biotin deficiency during normal pregnancy. *Am J Clin Nutr* **75**, 295-299
11) Coggeshall JC, Heggers JP, Robson MC, Baker H (1985) Biotin ststus and plasma glucose in diabetics. *Ann NY Acad Sci* **447**, 389-92
12) Reddi A, DeAngelis B, Frank O, Lasker N, Baker H (1988) Biotin supplementation improves glucose and insulin tolerances in genetically diabetic KK mice. *Life Sci* **42**, 1323-1330
13) Sone H, Ito M, Sugiyama K, Ohneda M, Maebashi M, Fukukawa Y (1999) Biotin enhances glucose-stimulated inslin secretion in the isolated perfused pancreas of the rat. *J Nutr Biochem* **10**, 237-243
14) Manthey KC, Griffin JB, Zempleni J (2002) Biotin supply affects expression of biotin transporters, biotinylation of carboxylases and metabolism of interleukin-2 in Jurkat cells. *J Nutr* **132**, 887-892
15) Couch JR, Cravent WW, Elvehjem CA, Halpin JG (1948) Relation of biotin to congenital deformities in the chick. *Anat Rec* **100**, 29-48
16) Whitehead CC (1980) Performance of laying hens fed on practical diets containing different levels of supplemental biotin during the rearing and laying stages. *Br J Nutr* **44**, 151-159
17) 奥村純市，藤原昇（2000）家禽学，pp40-70，朝倉書店，東京
18) 加藤嘉太郎（1974）家畜の解剖と生理，pp343-422，養賢堂，東京
19) 加藤嘉太郎（1969）家畜比較発生学，pp8-54，養賢堂，東京
20) 白井敏雄（1990）発生学，pp489-539，西村書店，東京
21) Zakaria AH, Miyaki T, Imai K (1984) The relationships of clutch length and egg position on ovarian follicular growth in laying hens. *Poult Sci* **63**, 1250-1254
22) Ronald RE, Landen WO (1998) Biotin : Vitamin Analysis for the Health and Food Sciences. pp478-487, CRC Press., Boca Raton
23) Ball GMF (2005) Microbiological methods for the determination of the B-group vitamins. Vitamins in Food. pp339-368, CRC Press, Boca Raton
24) Fukui T, Iinuma K, Oizumi J (1994) Agar plate method using *Lactobacillus plantarum* for biotin determination in serum and urine. *J Nutr Sci Vitaminol* **40**, 491-498
25) Hymes J, Wolf B (1996) Biotinidase and its roles in biotin metabolism. *Clin Chim Acta* **255**, 1-11
26) Wolf B (2005) Biotinidase: its role in biotinidase deficiency and biotin metabolism. *J Nutr Biochem* **16**, 441-445
27) Wolf B, Guier RE, Allen RJ, Goodman SI, Kien CL (1983) Biotinidase deficiency : the enzymatic defect in late-onset carboxylase deficiency. *Clin Chim Acta* **131**, 273-281
28) Watanabe T, Endo A (1984) Teratogenic effects of avidin-induced biotin deficiency in mice. *Teratology* **30**, 91-94
29) Harms RH, Damron BL, Simpson CF (1977) Effect of wet litter and supplemental biotin and/or whey on the production of foot pad dermatitis in broilers. *Poult Sci* **56**, 291-296
30) Cravens WW, Sebesta EE, Halpin JG, Hart EB (1942) Effect of biotin on reproduction in the domestic fowl. *Proc Soc Exp Biol Med* **50**, 101-104
31) Brewer LE, Edwards HM, Jr (1972) Studies on the biotin requirement of broiler breeders. *Poult Sci* **51**,619-624
32) Atuahene YO, Bernier PE, Roush WA, Arscott GH (1984) Effect of biotin on dermatitis and hatchability in dwarf and normal size single comb white leghorn layers. *Poult Sci* **63**,580-582
33) Bradly JW, Atkinson RL, Krueger WF (1976) Relationship of biotin to reproductive performance of leghorn-type hens. *Poult Sci* **55**, 2490-2492
34) Whitehead CC, Pearson RA, Herron KM (1985) Biotin requirements of broiler breeders fed diets of different protein content and effect of insufficient biotin on the viability of progeny. *Br Poult Sci* **26**, 73-82
35) Couch JR, Cravens WW, Elvehjem CA, Halpin JG (1948) Relation of carbohydrate to intestinal synthesis of biotin and hatchability in mature fowl. *J Nutr* **35**, 57-72

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Effects of Alpha Tocopherol and Probucol Supplements on Allergen-Induced Airway Inflammation and Hyperresponsiveness in a Mouse Model of Allergic Asthma

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Key Words
Asthma model mouse · α-Tocopherol · Probucol · Airway hyperresponsiveness · Cytokine · Oxidative stress

Abstract
Objective: We investigated the role of antioxidants in airway hyperresponsiveness to acetylcholine using young asthma model mice, which were sensitized and stimulated with ovalbumin. ***Methods:*** The mice had been fed either a normal diet, an α-tocopherol-supplemented diet or a probucol-supplemented diet 14 days before the first sensitization. They were immunized with antigen at intervals of 12 days and, starting from 10 days after the second immunization, they were exposed to antigen 3 times every 4th day using an ultrasonic nebulizer. Twenty-four hours after the last antigen inhalation, airway responsiveness to acetylcholine was measured and bronchoalveolar lavage fluid (BALF) was collected. A blood and lung tissue study was also carried out. ***Results:*** Twenty-four hours after the last antigen challenge, both IL-4 and IL-5 in the BALF of α-tocopherol-supplemented mice were significantly decreased. The IL-5 level in probucol-supplemented mice was also decreased, but there was no difference in IL-4 levels. The serum IgE level was decreased in probucol-supplemented mice. Differential cell rates of the fluid revealed a significant decrease in eosinophils due to antioxidant supplementation. Airway hyperresponsiveness to acetylcholine was also repressed in antioxidant-supplemented mice. In histological sections of lung tissue, inflammatory cells and mucus secretion were markedly reduced in antioxidant-supplemented mice. We investigated the antioxidant effect on our model mice by examining 8-isoprostane in BALF and lung tissue, and acrolein in BALF; however, our experiment gave us no evidence of the antioxidant properties of either α-tocopherol or probucol contributing to the reduction of airway inflammation. ***Conclusion:*** These findings indicate that α-tocopherol and probucol suppress allergic responses in asthma model mice, although these two drugs cause suppression in different ways that are unrelated to antioxidation.

Introduction

Asthma is a chronic inflammatory disease of the airways. The inflammatory cells (neutrophils, eosinophils, and macrophages) infiltrating the airways exist even in patients with mild asthma [1–3]. These cells release H_2O_2 or NO, resulting in the formation of superoxide or peroxynitrite anion [4–11], which are highly reactive oxygen species (ROS) that likely play a major role in asthma and are involved in cellular injury [12]. Increased levels of ROS are shown to be associated with many pathophysiologic states and characteristics of asthma, such as increased production of lipid peroxidation, increased air-

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Table 1. Composition of the normal diet

Ingredients	Concentration, %
Vitamin-free casein	20.0
DL-Methionine	0.3
Glucose	25.0
Sucrose	25.0
Alpha cornstarch	15.0
Cellulose	5.0
AIN-76 mineral mixture	1.0
AIN-76 vitamin mixture	3.5
Tartaric acid	0.2

The normal diet contains 20 mg α-TOC/kg diet. The α-TOC-supplemented diet was prepared by adding 480 mg α-TOC/kg diet to the normal diet and the 1% probucol diet was prepared by adding 1% v/w probucol to the normal diet. The composition is based on the AIN-76 diet.

way reactivity and secretion, increased production of chemoattractants, and increased vascular permeability [13]. Moreover, eosinophils, macrophages and neutrophils from asthmatic patients produce more ROS than those from normal subjects [14, 15]. On the other hand, changes in the antioxidant defenses of asthmatic patients have been reported, including decreased GSH-Px, selenium deficiency, decreased vitamins C and E, and decreased protein sulfhydrils and total antioxidant capacity in plasma [16–23]. As a result, the oxidant-antioxidant balance shifts toward increased oxidative stress in asthma. Some antioxidant therapies have previously been described as disappointing, but superoxide dismutases and N-acetylcysteine could attenuate the hyperresponsiveness of airways [24–26].

Vitamin E is a potent antioxidant that exists in nature and its relationship with immune functions is becoming clearer. Vitamin E can not only affect the metabolism of arachidonic acid via the 5-lipoxygenase pathway but also modulate the immune function directly through cellular and humoral immunity, and lymphoproliferative response at the thymus [27]. Concerning allergic diseases, it has been reported that vitamin E supplementation can reduce the symptoms and levels of serum immunoglobulin E (IgE) in a mouse model of nasal allergy [28], that there is a significant relationship between dietary vitamin E intake and serum IgE concentration in patients with atopic diseases [29], and that an injection of vitamin E reduced the airway hyperresponsiveness (AHR) of sensitized guinea pigs [30]. Vitamin E is therefore thought to improve the allergic state both as an antioxidant and immune modulator.

Probucol [4,4′-isopropylidenedithio-bis(2,6-di-tert-butylphenol)] is a lipophilic agent effective for hyperlipidemia and atherosclerosis, and is structurally different from other hypocholesterolemic agents. The antiatherogenic effects of probucol are known to relate not only to the hypocholestomic effect but also to the antioxidative effect [31–33]. In plasma, it is transported with lipoproteins, mainly by LDL, and prevents the oxidative modification of LDL by its radical-scavenging property [34]. Furthermore, probucol has a direct effect on macrophage migration without major impairment of the ability of the cells to accumulate and metabolize modified LDL. To clarify the effect of this potent antioxidant on asthma, we examined the possible usefulness of probucol to improve the AHR in a murine model compared with one species of vitamin E, α-tocopherol (α-TOC).

Materials and Methods

Animals

Five-week-old female BALB/C mice, weighing 16–20 g, were purchased from Crea Japan and housed in plastic cages in an air-conditioned room at 23 ± 2°C. The animals were divided into three groups (plus one control group) of 15 mice each. One group was fed a normal diet, one an α-TOC-supplemented diet and one a 1% probucol diet. Experiments were performed following the 1987 Japanese Association for Laboratory Animal Science guidelines for the care and use of experimental animals.

Diets

Normal and α-TOC-supplemented diets were purchased from Funabashi Farm (Chiba, Japan). The 1% probucol diet was made by mixing probucol (Daiichi Seiyaku, Tokyo, Japan) into a normal diet. The normal diet contains 20 mg α-TOC/kg diet and the α-TOC-supplemented diet originally contains 500 mg α-TOC/kg diet. All diets were based on AIN-76 diet (table 1) and prepared by being mixed with stripped corn oil (Tama Biochemical, Tokyo, Japan). The final concentration of corn oil was 9.1% v/w, which falls within the most recent recommended guidelines for the composition of AIN-93G. The diets were stored at −30°C before use. Fresh food was supplied each day.

Sensitization and Antigen Challenge

Figure 1 shows our experimental protocol. All three groups of mice were actively sensitized by intraperitoneal injections of 50 μg ovalbumin (OA; Nacalai Tesque, Kyoto, Japan) with 1 mg $Al(OH)_3$: alum (LSL, Tokyo, Japan) on day 14 and day 26. Starting on day 36, they were exposed to OA (1% w/v diluted in sterile saline) for 30 min, 3 times every 4th day according to a previously reported method by Tanaka et al. [35]. Control animals fed a normal diet were injected with 1 mg alum and sterile saline into the peritoneal cavity, and exposed to saline in a similar manner (hereinafter, this group is referred to as 'nonsensitized mice'). The

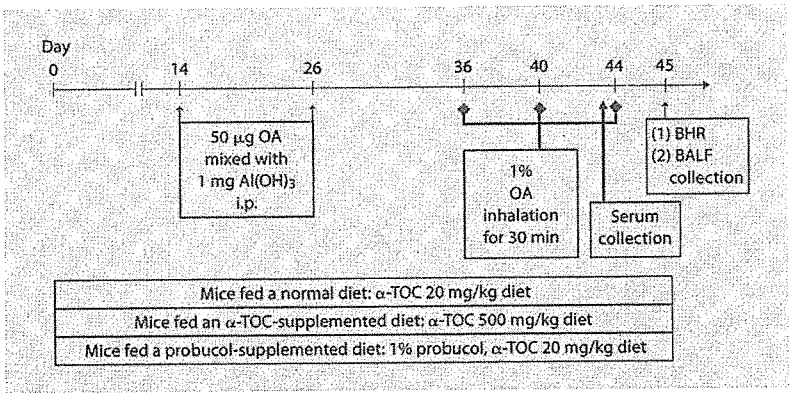

Fig. 1. Experimental protocol. Mice were fed either a normal diet, an α-TOC-supplemented diet or a probucol-supplemented diet. They were sensitized by intraperitoneal injections (i.p.) of 50 μg OA with 1 mg $Al(OH)_3$ on day 14 and day 26, and were exposed to 1% OA for 30 min on day 36 and then twice every 4th day. Nonsensitized mice were injected with $Al(OH)_3$ only and exposed to saline in a similar manner. Sera were obtained prior to the final antigen provocation. 24 h after the last antigen challenge, the mice were anesthetized and AHR to Ach was measured and BALF was obtained. BHR = Bronchial hyperresponsiveness.

aerosol (particle size: 1–8 μm) was generated using an ultrasonic nebulizer (NE-U12; Omron, Tokyo, Japan), which filled a cylindrical chamber (diameter 12 cm, height 17 cm), in which the mice were exposed.

Measurement of IgE

Just before the final allergen challenge, blood was collected from amputated tail ends. Sera were obtained by centrifugation and stored at −80°C. Values of IgE in mice sera were measured using the Rbis-IgE-ELISA kit for mice (Shibayagi, Gunma, Japan). Briefly, serum IgE was measured by coating monoclonal rat anti-mouse IgE antibodies. After being blocked with 1% BSA, serum dilutions were incubated for 2 h, then biotinylated-anti-mouse IgE antibodies and peroxidase-conjugated streptavidin were added. Sequentially diluted monoclonal mouse IgE antibodies were used as a standard. Optical densities of the enzymatic reactions were read using an automatic ELISA plate reader (Immuno Mini NJ-2300, Nalge Nunc Int., Tokyo, Japan) at 450 nm.

Detection of α-TOC and Probucol

Concentrations of α-TOC and probucol were detected using an HPLC system with an electrochemical detector (Shiseido, Tokyo, Japan). An LC-18 column (Supelco, Tokyo, Japan) was used with an eluent of methanol/$NaClO_4$ containing 50 mM $NaClO_4$ at a flow rate of 1 ml/min. The concentration of α-TOC was calculated by comparing the area under the curve (AUC) with that of an internal standard. The concentration of probucol was calculated from AUC using a standard curve [36].

Measurement of Airway Function

Measurement of bronchial responsiveness to acetylcholine chloride (Ach; Nacalai Tesque, Kyoto, Japan) was carried out according to a modified method previously described [35]. Ten of the 15 mice in each group were anesthetized with pentobarbital sodium salt (Tokyo Kasei, Tokyo, Japan; 80 mg/kg) and a 26-gauge needle was inserted into the jugular vein to inject several doses of Ach. Mice were injected with pancronium bromide (Sigma-Aldrich, St. Louis, Mo., USA; 0.1 mg/kg) intravenously to suppress spontaneous respiration. After disclosing the trachea, an 18-gauge cannula was inserted and the mice were ventilated with a rodent ventilator (SN-480-7; Shinano, Tokyo, Japan) at 0.2-ml tidal volume and 120 strokes/min. Bronchoconstriction was measured directly using a respiratory amplifier (AR-601G; Nihon Kohden, Tokyo, Japan) connected to the tracheal cannula. Changes in bronchial responsiveness to Ach were measured with doses of 62.5, 125, 250, 500, 1,000 and 2,000 μg/kg of Ach. We defined the area enclosed with the dose-response curve for Ach and each baseline of airway resistance as the AUC [35].

Bronchoalveolar Lavage Study

To evaluate the airway inflammation, we studied the accumulation of inflammatory cells and cytokines in the bronchoalveolar lavage fluid (BALF). After measuring airway function, the mice were anesthetized with intraperitoneal injections of pentobarbital sodium salt (150 mg/kg). We used calcium- and magnesium-free phosphate-buffered saline [PBS(−); Cosmo Bio Co., Tokyo, Japan] containing 0.1% bovine serum albumin (BSA; Biotecx Laboratories, Houston, Tex., USA) and 0.05 mM disodium ethylenediaminetetraacetic acid (EDTA-2Na; Nacalai Tesque, Kyoto, Japan) as a solution to collect the BALF. A cannula was inserted into the trachea and 1 ml solution was pumped in and out 4 times, sending it back and forth through the air lumen. This procedure was repeated 3 times (total volume, 3 ml) and about 90% of the injected solution was collected. The BALF of each animal was centrifuged at 1,500 rpm for 10 min. Cell pellets were floated in a 20% BSA solution (20 μl), then smeared onto slide glasses and stained with hematoxylin and eosin. Values of interleukin-4 (IL-4), interleukin-5 (IL-5) and interferon-γ (IFN-γ) in BALF were measured with ELISA kits (Endogen Mouse Interleukin-4 ELISA, Endogen Mouse Interleukin-5 ELISA, Mouse Interferon Gamma ELISA; Endogen, Cambridge, Mass., USA).

Histological Study

Five of 15 mice in each group were euthanized 24 h after the third antigen inhalation. Whole lungs were distended by 10% buffered formalin injected via the trachea. The tissues were then clamped at the trachea, excised from the bodies and immersed in the same fixative for 24 h. Tissues were sliced at 6-μm thickness, embedded in paraffin, and stained with hematoxylin and eosin, and Luna.

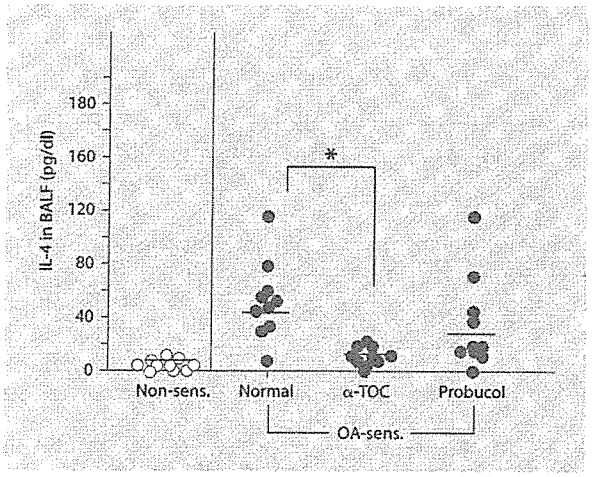

Fig. 2. IL-4 level in BALF was significantly increased by OA sensitization and exposure ($p < 0.005$). In mice fed an α-TOC diet, IL-4 level in BALF was decreased (* $p < 0.05$). Non-sens.: nonsensitized mice; normal: injected with saline and exposed to saline; OA-sens.: sensitized and exposed to OA.

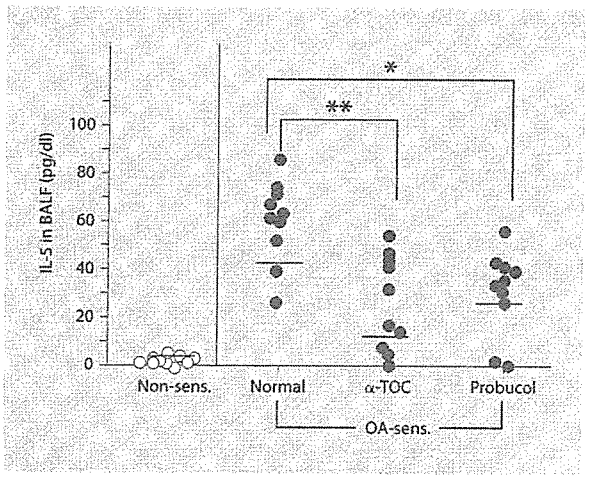

Fig. 3. IL-5 level in BALF was significantly increased by OA sensitization and exposure ($p < 0.05$). This level was decreased both in mice fed an α-TOC-supplemented and a probucol diet compared to mice fed a normal diet. * $p < 0.05$; ** $p < 0.001$.

Measurement of Oxidative Stress, 8-Isoprostane and Acrolein

Total 8-isoprostane values in BALF and lung tissue were quantified with an 8-isoprostane EIA kit (Cayman, Ann Arbor, Mich., USA). One milliliter of BALF or supernatant fluid of homogenized lung tissue in saline was incubated with KOH, and all 8-isoprostane was eluted to ethanol. The supernatant was diluted with ultrapure water and acidified with HCl. The samples were then extracted on C18 Sep-Pak cartridges (Waters, Milford, Mass., USA), which had been activated by rinsing first with methanol, then HCl of pH 3.0. After passing the samples through, the cartridges were rinsed first with HCl of pH 3.0, and then with heptane. The samples were eluted on anhydrous sodium sulfate (Wako, Osaka, Japan) with ethyl acetane-containing heptane, and extracted again on Silica Sep-Pak cartridges (Waters), which had been activated by rinsing first with methanol, then ethyl acetane. After the samples were passed through, the cartridges were rinsed again with ethyl acetane. Finally, purified 8-isoprostane was eluted with ethyl acetane-containing methanol. These solvents were evaporated and the samples were reconstituted with an assay buffer.

Acrolein (CH_2=CH–CHO; ACR) in BALF was measured with the ACR-lysine adduct ELISA system (NOF Medical Department, Tokyo, Japan).

Statistical Analyses

Data were presented as the mean ± SEM. Statistical analysis of data was performed by ANOVA. The significance of the difference between nonsensitized and OA-sensitized animals, and between animals on a normal diet and an antioxidant-supplemented diet sensitized by OA was estimated using Fisher's protected least significant difference. p values less than 0.05 were considered significant.

Results

Serum Concentration of α-TOC and Probucol

The mean concentrations of serum α-TOC in mice with normal diet and mice with α-TOC-supplemented diet were 213 ± 73 and 651 ± 67 μg/ml, respectively ($p < 0.001$). The mean concentration of serum probucol in mice receiving the probucol-supplemented diet was 33.7 ± 9.8 μg/ml, which is almost the same as that of humans who take probucol as medication.

IL-4, IL-5 and IFN-γ Levels in BALF

IL-4 levels in BALF of mice fed a normal diet were significantly increased by OA sensitization and exposure compared with nonsensitized mice ($p < 0.05$). Those of α-TOC-fed mice were significantly decreased compared with normal diet mice ($p < 0.05$) and, conversely, those of probucol-fed mice were not different from mice fed a normal diet and significantly increased compared with nonsensitized mice ($p < 0.05$) (fig. 2). IL-5 levels in BALF of mice fed a normal diet were significantly increased compared with nonsensitized mice ($p < 0.005$). Those of α-TOC-fed mice and probucol-fed mice were both significantly decreased compared with mice fed a normal diet ($p < 0.05$) (fig. 3). IFN-γ levels of mice fed a normal diet were significantly decreased compared with nonsensitized mice ($p < 0.0005$). There was no difference in the

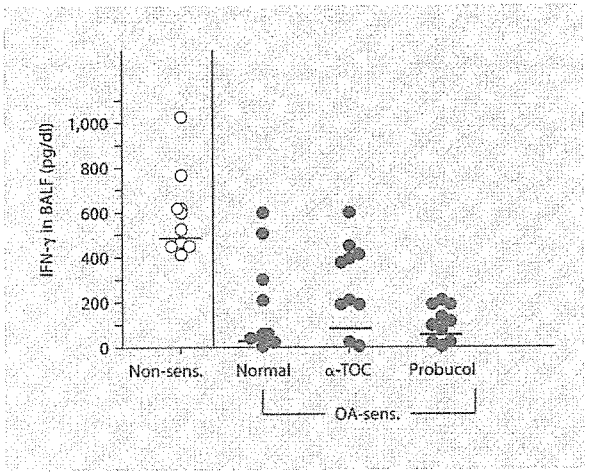

Fig. 4. IFN-γ level in BALF was decreased by OA sensitization and exposure ($p < 0.005$). There was no difference between levels in mice fed a normal, an α-TOC and a probucol diet.

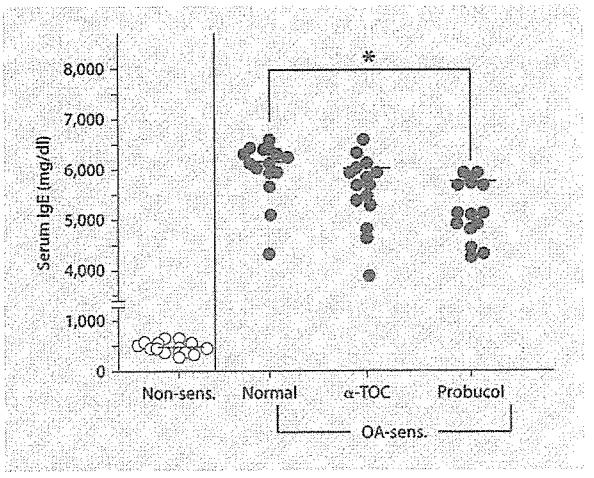

Fig. 5. Serum IgE level was increased by OA sensitization and exposure ($p < 0.005$). That of mice fed a probucol diet was significantly decreased compared with mice fed a normal diet. * $p < 0.05$.

IFN-γ levels of each diet group sensitized and exposed to OA and these were significantly decreased compared with nonsensitized mice (fig. 4).

Serum IgE Level

Serum IgE levels in mice fed a normal diet were significantly elevated by sensitization and exposure to OA compared with nonsensitized mice ($p < 0.0005$). Those of probucol-fed mice were significantly decreased ($p < 0.05$). There was no difference in the levels of mice fed a normal diet and α-TOC-fed mice (fig. 5).

Eosinophil Rates in BALF

Eosinophil rates in BALF of mice fed a normal diet were significantly increased by sensitization and exposure to OA compared with nonsensitized mice ($p < 0.0005$). Those of α-TOC-fed mice and probucol-fed mice were significantly decreased compared with mice fed a normal diet ($p < 0.005$) (fig. 6).

Airway Responsiveness to Ach

Figure 7 shows the dose-response curve of airway responsiveness to Ach after repeated antigen inhalation in each group. A curve of nonsensitized mice is also presented as a control and AHR was not found in these animals. The dose-response curve of airway responsiveness to Ach was shifted leftward in mice fed a normal diet after repeated allergen provocation and AUC was significantly increased compared with that of nonsensitized mice (943.61 ± 58.3 vs. 655.52 ± 17.4). The degree of airway responsiveness to Ach was smaller both in

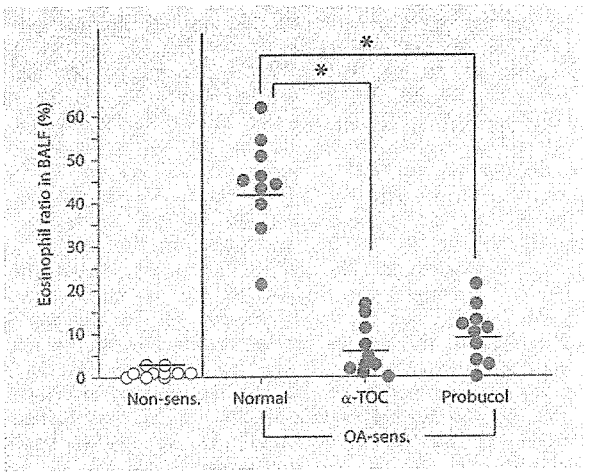

Fig. 6. Ratio of eosinophils to whole cells in BALF was significantly elevated by OA sensitization and exposure ($p < 0.005$). This ratio was decreased in both mice fed an α-TOC and a probucol diet. * $p < 0.05$.

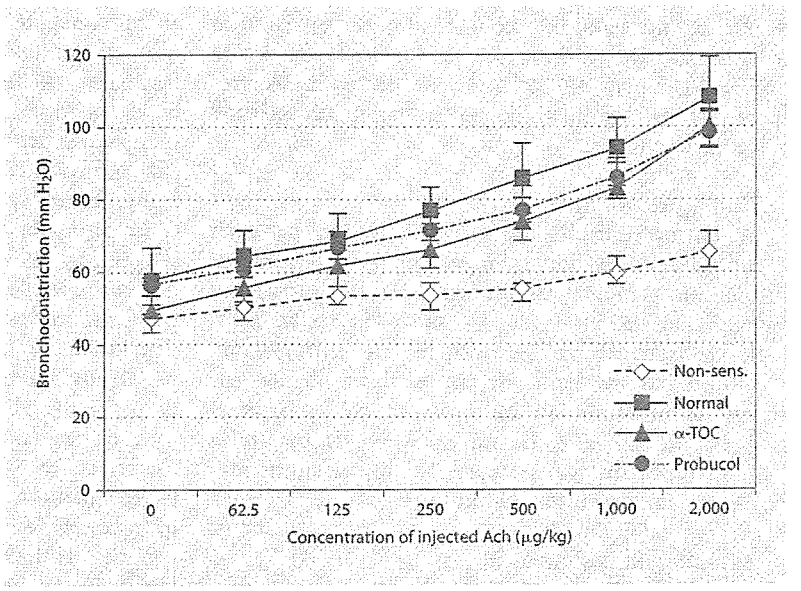

Fig. 7. Airway responsiveness to Ach 24 h after the final antigen challenge (mean ± SD). The AUC was calculated from the dose-response curve for Ach. AUC of mice fed a normal diet was significantly larger than that of nonsensitized mice ($p < 0.05$). AUC of mice fed an α-TOC-supplemented diet and mice fed a probucol diet was significantly smaller than that of mice fed a normal diet ($p < 0.05$).

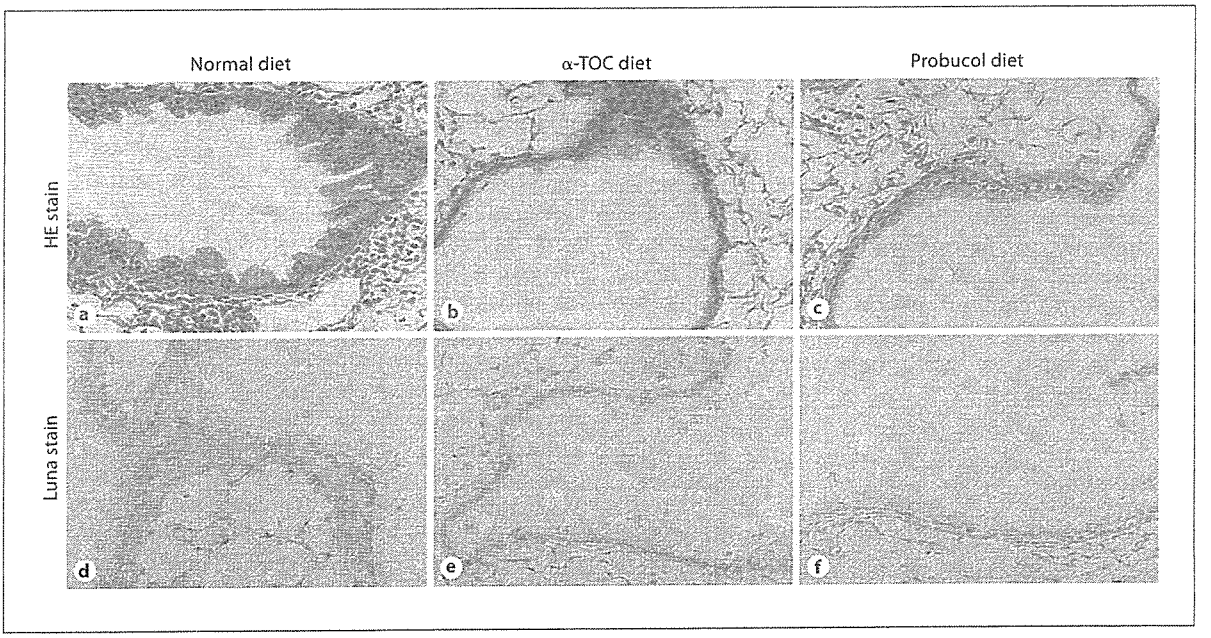

Fig. 8. Histological analysis of lung sections from mice fed a normal diet, an α-TOC diet and a probucol diet. Tissues were stained with hematoxylin and eosin (**a–c**) and with Luna (**d–f**). **a**, **d** Airway mucus hypersecretion and the infiltration of eosinophils were seen in mice fed a normal diet. **b**, **c**, **e**, **f** These findings were not detected in mice fed an α-TOC diet and a probucol diet. ×400.

α-TOC-fed mice and in probucol-fed mice than in mice fed a normal diet, and the AUC of α-TOC-fed mice and probucol-fed mice (827.89 ± 23.9 and 890.94 ± 38.2, respectively) was significantly smaller than that of mice fed a normal diet ($p < 0.05$).

Histological Analysis

We examined the histology of 5 of the 15 mice in each group and made 5 slices from each mouse. The lungs of nonsensitized mice showed normal histology (data not shown). Similar to the results in the BALF study, histological sections of lung tissue from mice fed a normal diet sensitized and provoked with OA exhibited obvious eosinophilic inflammation and airway mucus hypersecretion (fig. 8a, d). α-TOC-fed mice and probucol-fed mice had a marked reduction in airway inflammation and mucus secretion in their bronchiolus (fig. 8b, c, e, f).

8-Isoprostane and Acrolein

The levels of 8-isoprostane in BALF of nonsensitized, normal-diet, α-TOC and probucol group were 366.69 ± 107.44, 248.24 ± 177.32, 276.54 ± 61.21 and 264.61 ± 51.99 pg/ml, respectively. There was no difference between sensitized mice groups. The levels of 8-isoprostane in lung tissue of the nonsensitized, normal-diet, α-TOC and probucol group were 447.77 ± 44.00, 1,629.37 ± 164.44, 1,716.75 ± 395.80 and 1,843.904 ± 272.88 pg/mg, respectively. Those of sensitized mice were significantly increased compared with that of nonsensitized mice ($p < 0.05$), but there was no difference between all three sensitized groups. The levels of acrolein in BALF of the nonsensitized, normal-diet, α-TOC and probucol group were 27.74 ± 5.88, 48.93 ± 8.54, 50.48 ± 16.10 and 52.49 ± 14.42 nmol/ml, respectively. These results are statistically almost identical between the three sensitized groups, though it was significantly increased by OA sensitization ($p < 0.05$).

Discussion

In our study, we found that repeated exposure of a sensitized animal to antigen caused a significant increase in several markers of airway inflammation and bronchial responsiveness to Ach, and that these effects were inhibited by both α-TOC and probucol supplements; however, some factors were different in each group. Eosinophil infiltration in BALF and lungs, the values of IL-5 in BALF and serum IgE levels after the final antigen provocation were all significantly decreased in both α-TOC-fed mice and probucol-fed mice in comparison with mice fed a normal diet. On the other hand, the values of IL-4 in BALF after the final antigen inhalation were significantly decreased in α-TOC-fed mice, and were significantly increased in probucol-fed mice in comparison with mice fed a normal diet. These results indicate that both α-TOC and probucol, which are known to relieve oxidative stress, could also decrease airway inflammation and AHR in sensitized animals. However, we were unable to find any difference in 8-isoprostane and acrolein levels between the three groups. 8-Isoprostanes are arachidonate products formed on membrane phospholipids by the activity of ROS and represent a quantitative measurement of oxidant stress in vivo [37]. Acrolein has a strong cytotoxicity and is also increased by lipid oxidation. We expected the effects of α-TOC and probucol as antioxidants on asthma model mice; however, our experiment gave us no evidence of the antioxidant properties of these drugs contributing to the reduction of airway inflammation. This fact suggests that these drugs could influence the immune system directly (although in different ways), and not merely through the oxidative pathway. Further studies into the usefulness of these products for asthma model mice should be performed.

There have been a few recent reports indicating that α-TOC has a modulating effect on the immune system. Zheng et al. [28] reported that higher doses of vitamin E supplementation may suppress both nasal allergic responses and the increase of serum IgE in nasal allergy model mice sensitized by TDI. They investigated splenocyte proliferation and cytokine production from splenocytes, and found that vitamin E could influence the immune responses directly. Fogarty et al. [29] investigated the relationship between dietary vitamin E intake and serum IgE concentrations and atopy in a random sample of 2,633 adults. They reported that higher concentrations of vitamin E intake were associated with lower serum IgE concentrations and a lower frequency of allergen sensitization. Tsourei-Nikita et al. [38] investigated the dietary intake of vitamin E in patients with atopic dermatitis and found that there were negative correlations between vitamin E intake, and both serum IgE levels and clinical manifestations. Initially, vitamin E, which is a potent antioxidant, exhibited the ability to enhance both cellular and humoral immunity in vitro and in vivo. The results of our study and former findings suggest that vitamin E can play an important role in IgE-mediated atopic responses to allergic stimuli by decreasing the serum IgE levels. For this reason, vitamin E is now thought to be an immune regulator.

Although serum IgE levels in mice fed an α-TOC diet after the final antigen challenge did not differ from those in mice fed a normal diet, other markers (such as IL-4 and IL-5 levels in BALF, eosinophil count in BALF, inflammation of lung tissue and airway responsiveness to Ach) were decreased significantly compared to those in mice fed a normal diet. This discrepancy is possibly due to OA provocation being stronger than the inhibitory effect of α-TOC and/or the fact that α-TOC affects the allergic pathways further from the IgE-mediated response. Another possibility is that α-TOC and probucol each affect a different point of the allergic pathway from Th2 cells to eosinophilic inflammation. For example, IL-5 is produced by both Th2 cells and eosinophils, but IL-4 is produced only by Th2 cells. This might explain why IL-4 levels in BALF after the final antigen challenge differ between α-TOC-fed mice and probucol-fed mice.

There is increasing evidence that inflammation from asthma results in increased oxidative stress in the airways. The H_2O_2 concentration and NO levels in exhaled air condensate are increased in stable asthmatics and they may contribute to airway edema and inflammation [39–41]. The inflammatory and immune cells in the airways (such as macrophages, neutrophils and eosinophils) release increased amounts of ROS in asthmatic patients [15, 42], and their ability to produce O_2^- correlates with the degree of bronchial responsiveness to inhaled methacholine [43]. The direct oxidative damage can result in the characteristic features of asthma [44, 45] and it may evoke AHR [46]. Leukotrienes (LT) are now thought to play an important role in AHR in asthma [1] and oxidative stress leads to AHR via LT production [47]. Centanni et al. [48] studied the effect of TOC supplementation and the results obtained showed a significant inhibition of LT production by α-TOC supplementation. These studies provide support for the hypothesis of a potential effect of α-TOC supplementation in asthma patients. As the results of our experiment, we confirmed that allergic response leads to oxidative stress in the airways of mice. However, there was no evidence of the antioxidant properties of α-TOC. We suggest that the correlation of the antioxidant dose with inflammatory intensity may contribute to this discrepancy between previous reports and our results; however, the details of these mechanisms remain unknown.

On the other hand, the effect of probucol on allergic diseases has not yet been clarified. Probucol is a cholesterol-lowering drug, and serum cholesterol increases in asthmatic patients [49]. Yeh and Huang [50] reported that dietary cholesterol enhances bronchial inflammation in a murine model of asthma. While probucol is used as an antioxidant in various diseases [51], a recent study showed that the immunomodulatory effect of probucol predicts a role in chronic inflammatory diseases [52]. This immunomodulatory effect is believed to be independent of the cholesterol-lowering effect. We believe that the pleiotropic effects of probucol outlined above are responsible for the differences in results between the α-TOC and probucol groups. From our study of AHR in animal models, we concluded that probucol may also have a potent effect on IgE-mediated atopic responses and reduces serum IgE levels.

In our study, α-TOC and probucol also suppressed AHR in asthma model mice, although they used different immunological pathways against Th2 activity and/or the IgE-mediated allergic response. Additional studies are needed to clarify the mechanism of each activity and its potential role for the treatment of asthmatic patients. The optimal dose of each drug should also be determined.

References

1 Adkinson NF Jr, Yunginger JW, Busse WW, Bochner BS, Holgate ST, Simons FER: Middleton's Allergy: Principles & Practice, ed 6. Philadelphia, Mosby, 2003.

2 Sedgwick JB, Geiger KM, Busse WW: Superoxide generation by hypodense eosinophils from patients with asthma. Am Rev Respir Dis 1990;142:120–125.

3 Emelyanov A, Fedoseev G, Abulimity A, Rudinski K, Fedoulov A, Karabanov A, et al: Elevated concentration of exhaled hydrogen peroxide in asthmatic patients. Chest 2001; 120:1136–1139.

4 Zanconato S, Scollo M, Zaramella C, Landi L, Zacchello F, Baraldi E: Exhaled carbon monoxide levels after a course of oral prednisone in children with asthma exacerbation. J Allergy Clin Immunol 2002;109:440–445.

5 Hanazawa T, Kharitonov SA, Barnes PJ: Increased nitrotyrosine in exhaled breath condensate of patients with asthma. Am J Respir Crit Care Med 2000;162:1273–1276.

6 Shanmugasundaram KR, Kumar SS, Rajajee S: Excessive free radical generation in the blood of children suffering from asthma. Clin Chim Acta 2001;305:107–114.

7 Chanez P, Dent G, Yukawa T, Bernes PJ, Chang KF: Generation of oxygen free radicals from blood eosinophils from asthma patients after stimulation with PAF or phorbol ester. Eur Respir J 1990;3:1002–1007.

8 Cluzel M, Damon M, Chanaz P, Bonsquet J, Crastesde PA, Michel FB, et al: Enhanced alveolar cell luminol-dependent chemiluminescence in asthma. J Allergy Clin Immunol 1987;80:195–201.

9 Calhoun WJ, Salisbury SM, Bush RK, Busse WW: Increased superoxide release from alveolar macrophages in symptomatic asthma. Am Rev Respir Dis 1987;135:A224.

10 Vachier I, Damon M, Le Doucen C, Crastes-de PA, Chanez P, Michel FB, et al: Increased oxygen species generation in blood monocytes of asthma patients. Am Rev Respir Dis 1992;146:1161–1166.
11 Warke TJ, Fitch PS, Brown V, Taylor R, Lyons JDM, Ennis M, et al: Exhaled nitric oxide correlates with airway eosinophils in childhood asthma. Thorax 2002;57:383–387.
12 Michalec L, Choudhury BK, Postlethwait E, Wild JS, Alam R, Brown ML, et al: CCL7 and CXCL10 orchestrate oxidative stress-induced neutrophilic lung inflammation. J Immunol 2002;168:846–852.
13 Barnes PJ: Reactive oxygen species and airway inflammation. Free Radic Biol Med 1990;9:235–243.
14 Nadeem A, Chhabra SK, Masood A, Hanumantharao GR: Increased oxidative stress and altered levels of antioxidants in asthma. J Allergy Clin Immunol 2003;111:72–78.
15 Schauer U, Leinhaas C, Jager R, Rieger CH: Enhanced superoxide generation by eosinophils from asthmatic children. Int Arch Allergy Appl Immunol 1991;96:317–321.
16 Rahman I, Morrison D, Donaldson K, MacNee W: Systemic oxidative stress in asthma, COPD and smokers. Am J Respir Crit Care Med 1996;159:1055–1060.
17 Hesselmark OL, Malmgren R, Unge G, Zetterstorm O: Lowered platelet GSH-Px activity in patients with intrinsic asthma. Allergy 1990;45:523–527.
18 Pearson DJ, Saurez-Mendez VJ, Day JP, Miller PF: Selenium status in relation to reduced glutathione peroxidase activity in aspirin sensitive asthma. Clin Exp Allergy 1991;21:203–208.
19 Picado C, Deulofen R, Lieonart R, Agusti M, Mullol J, Quinto L, et al: Dietary micronutrients/antioxidants and their relationship with bronchial asthma severity. Allergy 2001;56:43–49.
20 Troisi RJ, Willett WC, Weiss ST, Trichopoulos D, Rosner B, Speizer FE: A prospective study of diet and adult-onset asthma. Am J Respir Crit Care Med 1995;151:1401–1408.
21 Hijazi N, Abalkhail B, Seaton A: Diet and childhood asthma in a society in transition: a study in urban and rural Saudi Arabia. Thorax 2000;55:775–779.
22 Kelly FJ, Mudway I, Blomberg A, Frew A, Sandstrom T: Altered lung antioxidant status in patients with mild asthma. Lancet 1999;354:482–483.
23 Kalayci O, Besler T, Kilinc K, Sekerel BE, Saraclar Y: Serum levels of antioxidant vitamins (alpha tocopherol, beta carotene and ascorbic acid) in children with bronchial asthma. Turk J Pediatr 2000;42:17–21.
24 Romieu I, Sien-Monge JJ, Ramirez-Aguilar M, Tellez-Rojo MM, Moreno-Macias H, Reyes-Ruiz NI, et al: Antioxidant supplementation in lung function among children with asthma exposed to high levels of air pollutants. Am J Respir Crit Care Med 2002;166:703–709.
25 Katsumata U, Miura M, Kimura K, Takahashi T, Inoue H, Takishima T: Oxygen radicals produce airway constriction and hyperresponsiveness in anesthetized cats. Am Rev Respir Dis 1990;141:1158.
26 Stey C, Steurer J, Bachmamm S, Medici TC, Tramer MR: The effect of oral N-acetylcysteine in chronic bronchitis. Eur Respir J 2000;16:253–262.
27 Moriguti S, Muraga M: Vitamin E and immunity. Vitam Horm 2000;59:305–336.
28 Zheng K, Adjei AA, Shinjo M, Shinjo S, Todoroki H, Ariizumi M: Effect of dietary vitamin E supplementation on murine nasal allergy. Am J Med Sci 1999;318:49–54.
29 Fogarty A, Lewis S, Weiss S, Britton J: Dietary vitamin E, IgE concentrations, and atopy. Lancet 2000;356:1573–1574.
30 Kilic FS, Erol K: The effect of vitamin E in ovalbumin-sensitized guinea pigs. Methods Find Exp Clin Pharmacol 2003;25:27–31.
31 Carew TE, Schwenke DC, Steinberg D: Antiatherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants in vivo can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit. Proc Natl Acad Sci USA 1987;84:7725–7729.
32 Keaney JF, Xu A, Cunningham D, Jackson T, Frei B, Vita JA: Dietary probucol preserves endothelial function in cholesterol-fed rabbits by limiting vascular oxidative stress and superoxide generation. J Clin Invest 1995;95:2520–2529.
33 Gorogawa S, Kajimoto Y, Umayahara Y, Kaneto H, Watada H, Kuroda A, et al: Probucol preserves pancreatic beta-cell function through reduction of oxidative stress in type 2 diabetes. Diabetes Res Clin Pract 2002;57:1–10.
34 Trach CC, Wulfroth PM, Severs NJ, Robenek H: Influence of native and modified lipoproteins on migration of mouse peritoneal macrophages and the effect of the antioxidants vitamin E and probucol. Eur J Cell Biol 1996;71:199–205.
35 Tanaka H, Kawada N, Yamada T, Kawada K, Takatsu K, Nagai H: Allergen-induced airway inflammation and bronchial responsiveness in interleukin-5 receptor alpha chain-deficient mice. Clin Exp Allergy 2000;30:874–881.
36 Kudo S, Akiyama H, Odomi M, Miyamoto G: High-performance liquid chromatographic procedure for the determination of probucol in human plasma. J Chromatogr 1983;277:419–422.
37 Dworski R, Murray JJ, Roberts LJ, Oates JA, Morrow JD, Fisher L, et al: Allergen-induced synthesis of F(2)-isoprostanes in atopic asthmatics. Evidence for oxidant stress. Am J Respir Crit Care Med 1999;160:1947–1951.
38 Tsourei-Nikita E, Hercogova J, Lotti T: Evaluation of dietary intake of vitamin E in the treatment of atopic dermatitis: a study of the clinical course and evaluation of the immunoglobulin E serum levels. Int J Dermatol 2002;41:146–150.
39 Comhair SA, Bhathena PR, Dweik RA, Kavuru M, Erzurum SC: Rapid loss of superoxide dismutase activity during antigen-induced asthmatic response. Lancet 2000;355:624.
40 Silkoff PE, Sylvester JT, Zamel N, Permutt S: Airway nitric oxide diffusion in asthma: role in pulmonary function and bronchial responsiveness. Am J Respir Crit Care Med 2000;161:1218–1228.
41 Ashutosh K: Nitric oxide and asthma: a review. Curr Opin Pulm Med 2000;6:21–25.
42 Calhoun WJ, Reed HE, Moest DR, Stevens CA: Enhanced superoxide production by alveolar macrophages and air-space cells, airway inflammation, and alveolar macrophage density changes after segmental antigen bronchoprovocation in allergic subjects. Am Rev Respir Dis 1992;145:317–325.
43 Seltzer J, Bigby BG, Stulbarg M, Holtzman MJ, Nadel JA, Ueki IF, et al: O_3-induced change in bronchial reactivity to methacholine and airway inflammation in humans. J Appl Physiol 1986;60:1321–1326.
44 Hulsmann AR, Raatgeep HR, den Hollander JC, Stijnen T, Saxena PR, Kerrebijn KF, et al: Oxidative epithelial damage produces hyperresposiveness of human peripheral airways. Am J Respir Crit Care Med 1994;149:519–525.
45 Krishna MT, Madden J, Teran LM, Biscione GL, Lau LC, Withers NJ, et al: Effects of 0.2 ppm ozone on biomarkers of inflammation in bronchoalveolar lavage fluid and bronchial mucosa of healthy subjects. Eur Respir J 1998;11:1294–1300.
46 Cortijo J, Marti-Cabrera M, de la Asuncion JG, Pallardo FV, Esteras A, Bruseghini L, et al: Contraction of human airways by oxidative stress protection by N-acetylcysteine. Free Radic Biol Med 1999;27:392–400.
47 Rouzer CA, Matsumoto T, Samuelsson B: Single protein from human leukocytes possesses 5-lipoxygenase and leukotoriene A4 synthase activities. Proc Natl Acad Sci USA 1986;83:857–861.
48 Centanni S, Santus P, Marco FD, Fumagalli F, Zarini S, Sala A: The potential role of tocopherol in asthma and allergies. BioDrugs 2001;15:81–86.
49 Misso NL, Brooks-Wildhaber J, Ray S, Vally H, Tompson PJ: Plasma concentrations of dietary and nondietary antioxidants are low in severe asthma. Eur Respir J 2005;26:257–264.
50 Yeh YF, Huang SL: Dietary cholesterol enhances pulmonary eosinophilic inflammation in a murine model of asthma. Int Arch Allergy Immunol 2001;125:329–334.
51 Kaneto H, Kawamori D, Matsuoka TA, Kajimoto Y, Yamasaki Y: Oxidative stress and pancreatic beta-cell dysfunction. Am J Ther 2005;12:529–533.
52 Doggrell SA: Immunomodulation with Di-NAC – a new approach to the treatment of atherosclerosis? Expert Opin Investig Drugs 2002;11:717–720.