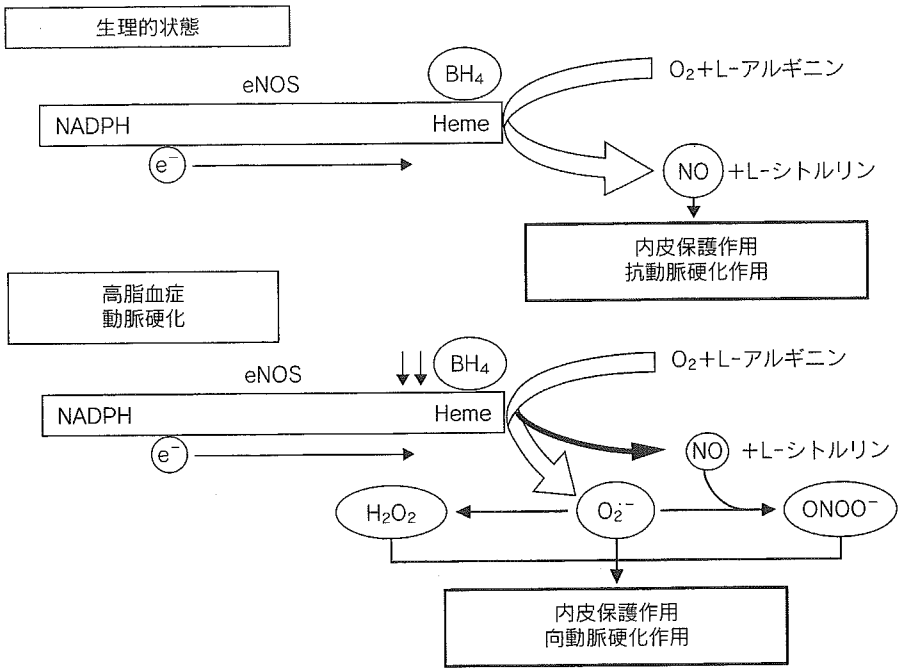


図❶ eNOS の動脈硬化における二面性（われわれの仮説）
高脂血症・動脈硬化では補酵素 BH_4の含有量低下により eNOS uncoupling が生じる．eNOS は NO のみならず $O_2^{\cdot-}$も産生し，NO の抗動脈硬化作用を阻害するとともに，場合によっては動脈硬化の悪化に関与する可能性がある．
(Kawashima S *et al*：*Arterioscler Thromb Vasc Biol* **24**：998-1005, 2004 より引用改変)

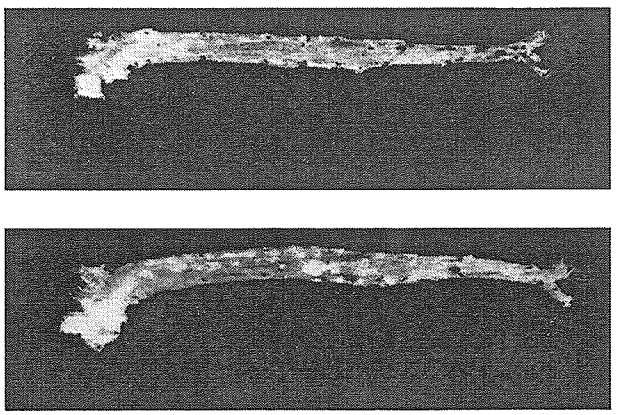
その後の追跡冠動脈造影でアセチルコリンにより血管が拡張せずに収縮した部位，すなわち内皮が傷害され EDR が減弱している部位に，器質的狭窄病変が出現したと述べられている．そして，内皮機能のうち最も重要なものが eNOS 由来の NO 産生であることより，これら一連の臨床研究の結果は，eNOS 由来 NO が，動脈硬化の防止・抑制にはたらいていることを示唆する所見と理解される．

動脈硬化に対して eNOS は抑制作用をもたらすだけなのか？

すでに NOS の遺伝子操作マウスの項で述べたように，eNOS 遺伝子の欠損が逆に動脈硬化の抑制をもたらすとの報告[14]も存在する．この報告は何を意味するのだろうか？ この報告ではその機序について明記はしていないものの，コントロールマウスでは内皮細胞による LDL 酸化が生じており，それは NOS 阻害薬でブロックされるのに対し，eNOS 遺伝子欠損マウスの内皮細胞による LDL 酸化は軽度で，しかも NOS 阻害薬でブロックできなかったと報告されている．これは eNOS が LDL の酸化に関与していることを示唆するものである．

この数年 NOS 研究において“NOS uncoupling（機能異常）”という現象が注目されている[22)23)]．これは NOS がある種の条件下では，NO よりむしろ $O_2^{\cdot-}$を産生するということを意味する．すなわち，NOS が NO 産生酵素として正常に作用するには，補酵素であるテトラヒドロビオプテリン（BH_4）の存在が必須であり，BH_4の不足下では NOS はダイマー化できず不安定となり，NOS のリダクターゼ領域で産生された電子が，オキシゲナーゼ領域において L-アルギニンよりむしろ酸素に供与される結果，$O_2^{\cdot-}$が産生される[24)]．

生体においても，この NOS uncoupling はまず糖尿病における内皮機能障害に関係していることが報告され[25)26)]注目された．その後，2001 年に動脈硬化血管においても



図❷ apoE 遺伝子欠損マウスでは eNOS 過剰発現により奇異性に動脈硬化病変が増強する（16 週齢での大動脈における検討）
A：通常の apoE 遺伝子欠損マウスの動脈硬化病変，
B：eNOS を過剰発現した apoE 遺伝子欠損マウスの動脈硬化病変．
（Ozaki M *et al*, 2002[29] より引用）

同様の現象が生じており，動脈硬化血管における EDR の減弱に関与していることがはじめて報告された[27]（図❶）．

同時期に，われわれは内皮細胞に eNOS が過剰発現するトランスジェニックマウス（eNOS-Tg）[28] と，動脈硬化易発症モデルである apoE 遺伝子欠損マウス（apoE-KO）との交配実験をおこなっており，その結果，当初の予想に反し，eNOS を過剰発現させた apo E 遺伝子欠損マウス（apo E-KO/eNOS-Tg）では，apoE-KO にくらべ動脈硬化病変は減少しておらず，逆に増強していることを見出していた（図❷）．

そこでわれわれ[29]は，この奇異性の動脈硬化病変増強の機序として，eNOS の uncoupling による内皮からの $O_2^{\cdot-}$ 産生の増加が関係すると考え，更なる検討をおこなった．そして内皮での eNOS 過剰発現の結果，血管壁，とくに内皮からの $O_2^{\cdot-}$ 産生が増加していること，そして血管における BH_4 濃度が低下していることを見出した．この動脈硬化血管における BH_4 濃度の低下は，BH_4 合成の減少と BH_4 が酸化され BH_2 に変化することの 2 つの機序が関係する．そこで合成 BH_4 の長期経口投与おこなうと，血管壁の BH_4 濃度の増加とともに，eNOS 過剰発現により増強した動脈硬化病変が，ほぼ通常の apoE 遺伝子欠損マウスの病変レベルまで軽減することを見出した．また，さらに内因性の BH_4 濃度を増加させるため，BH_4 産生の律速酵素である GTPCH-1（GTP cyclohydrolase-1）が内皮に過剰発現した遺伝子操作マウスとの交配実験をおこない，GTPCH-1 が過剰発現した apoE-KO/eNOS-Tg (apoE-KO/eNOS-Tg/GTPCH-Tg) を作製した．その結果，apoE-KO/eNOS-Tg/GTPCH-Tg では，血管壁の BH_4 濃度の増加とともに，血管壁からの $O_2^{\cdot-}$ 産生が低下し，動脈硬化病変が軽減することを見出した．

以上の一連の研究より，高脂血症・動脈硬化では補酵素 BH_4 の低下のため，過剰発現させた eNOS には uncoupling が生じており，eNOS は NO よりむしろ $O_2^{\cdot-}$ を産生していること，この増加した $O_2^{\cdot-}$ により動脈硬化が増強したものと考えられた．また，通常の apoE 遺伝子欠損マウスにおいても eNOS uncoupling が生じており，この uncoupling を BH_4 を増加させることにより是正すると，動脈硬化病変が軽減することも報告された[30]．その後の研究により，内皮細胞において eNOS が NO 産生酵素として最も効率的にはたらくためには，至適な eNOS と BH_4 のバランスが存在しており，そのバランスが少しでも BH_4 の相対的不足に傾くと，NOS 産生酵素としての活性が低下することも明らかになりつつある[31]．以上をふまえてわれわれは現在，eNOS 過剰発現マウスで見出した動脈硬化病変の増強は，特殊な条件下の研究であるものの，eNOS uncoupling が，本来の eNOS/NO 系の抗動脈硬化作用を減弱させているという現象は，おそらくヒトの動脈硬化の進展プロセスにおいても生じているのではないかと考えている．

このような観点に立つと，動脈硬化を eNOS/NO 系から防止，治療しようと考えるとき，単に eNOS 発現を増強させるだけでなく，eNOS の NO 産生酵素としての作用を最大限に引き出すため，並行して内皮細胞における BH_4 濃度を増加させるような治療法が望まれることになる．BH_4 の内皮細胞での代謝経路やその産生を規定する因子に関しては詳細はまだ明らかでない．同様に BH_4 の主たる産生酵素 GTPCH-1 の活性や発現の調節因子にも不明な点が多い．一方，動脈硬化の抑制効果が確立しているスタチンに，GTPCH-1 の発現増加，BH_4 の産生増

加作用があることが明らかになっている[32]．この意味においても，高脂血症・動脈硬化においてスタチンを投与することは非常に合目的であり，他の薬剤のBH_4に対する影響に関しての知見を広げるとともに，BH_4を効率的に増加させる薬剤の開発が望まれる．

おわりに

このようにeNOSにより産生されるNOは抗動脈硬化分子としてはたらくと考えられるが，eNOSが必ずしもNOのみを産生するのではなく，そのカウンター分子であるO_2^-をも産生することとは，eNOS/NO系を用いた動脈硬化の治療法を考えるうえで留意すべきことである．また他のNOS，すなわちiNOSあるいはnNOSにより産生されるNOが，生体において実際にどのように動脈硬化と関係しているかに関してはまだ不明な点も多く，更なる今後の研究が望まれる．

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特集：動脈硬化性疾患の臨床の今後の展望—❸

「動脈硬化・動脈硬化性疾患の薬物治療の今後の展開」

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【キーワード：コレステロール、メタボリックシンドローム、内皮障害、炎症、免疫療法】

●1. はじめに

スタチンの開発により飛躍的に動脈硬化、動脈硬化性疾患の発症防止を目的とした薬物治療は進んだといえよう。しかしながらそれにもかかわらず、食生活や生活習慣の変化、高齢化などの要因により、ますます冠動脈疾患をはじめとした動脈硬化性疾患は増加してきている。また、スタチンを例にとると心血管事故の発症は30%程度抑制できるが、逆に言えば残りの70%は発症するわけであり、動脈硬化性疾患に対する新たな薬物治療の開発が必要となっている。動脈硬化性疾患の治療においては、その基盤にある動脈硬化、とくに粥状硬化症の防止と、急性冠症候群における粥腫の破綻などのそれぞれの疾患の引き金となるイベントの防止の2点から考える必要がある。この中でより重要なのは、やはり基盤となる動脈硬化そのものの防止、治療であり、現在の薬物治療の中心はスタチンをはじめとしたLDLコレステロールに焦点を当てた治療法となっている。そして、将来の治療戦略としては、コレステロールをはじめとした脂質代謝の観点からの治療法と、炎症、免疫の制御の観点からの治療法の二つが重要と考えられる。本稿では、動脈硬化性疾患の基盤にある、動脈硬化そのものに焦点を当てた薬物治療の今後の展開について述べてみたい。

●2. コレステロールの観点からの治療

現在の動脈硬化治療の中心となっているのは、LDLコレステロールに焦点を当てたスタチンをはじめとした治療法であり、高LDL血症は血管壁に蓄積するコレステロールの供給源となるだけでなく、酸化LDLが動脈硬化の過程に関係する様々な生物作用を有することより、この高LDL血症を治療することは非常に合目的である。既にわが国でも5種類のスタチンが保険適応となっており、数多くの大規模臨床研究により、動脈硬化性疾患の発展防止におけるその有用性は確立し、現在も新しい臨床でのエビデンスが次々と報告されつつある。

スタチンに関し、今後更に明らかにしていかなくてはならない問題として、既に抗動脈硬化薬として、そのコレステロール低下作用による抗動脈硬化作用、心血管事故抑制作用は確立したものであるが、コレステロール低下作用に依存しない、いわゆるpleiotropicな作用がどの程度重要であるかということがまずあげられる。そして、現在、心血管事故抑制において、コレステロール低下に関しては“the lower, the better”との考えが、欧米においては、大規模臨床試験の結果をもとに広がりつつある。しかしながら、わが国においてスタチンの大規模臨床試験はほとんど行われておらず、わが国でのエビデンスの構築と共に、医療経済、費用対効果の視点からの取り組みも必要になってくると思われる。

LDLコレステロール低下の観点からの新たな治療法としては、コレステロール吸収阻害薬（Ezetimibe）が、2005年度の夏にはわが国でも保険適応となる見込みである。脂質の摂取が多い欧米で既に臨床に用いられており、その作用機序に関してはまだ不明な点が多いものの、小腸での食餌中および胆汁中のコレステロール吸収を阻害し、トリグリセリドや脂溶性ビタミンの吸収には影響を与えない。スタチンのみでは十分なLDLコレステロールの低下が得られない症例に、スタチンと併用すると、コレステロールの合成と吸収の両面を阻害することにより、効果的なコレステロール低下作用が得られるとされている。スタチン増量に伴う副作用防止の観点からも有用であろう。

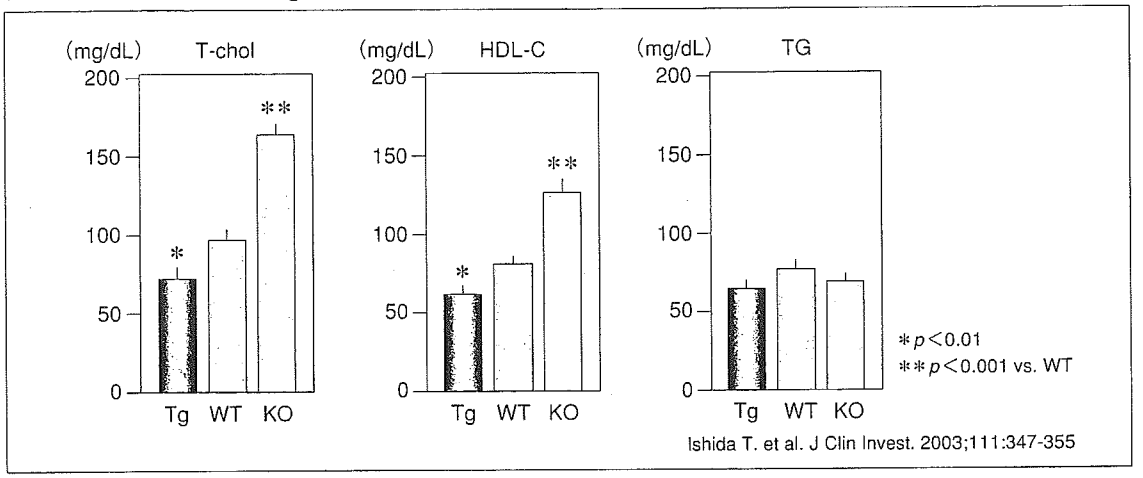
また、コレステロールの観点からでは、高LDL血症とならび重要なのは低HDL血症である。特にわが国では、高LDL血症よりむしろ低HDL血症が危険因子として問題となっている。HDLによる動脈硬化の防御機構のうち、最も重要なのは、動脈硬化巣に蓄積した余剰なコレステロールを引き抜き、肝臓へと転送するコレステロール逆転送（reverse cholesterol transport）である。HDLにより末梢組織から引き抜かれた遊離コレステロールは、HDL粒子中でLCAT（lecithin: cholesterol acyltransferase）によりエステル化され、コレステロールエステル（CE）となる。CEはHDLの中心部に取り込まれ、その後肝臓に転送され、胆汁酸へと代謝・処理されるが、肝臓にCEが取り込まれるには3種類の過程がある。すなわちHDL粒子自身が取り込まれる過程、CEが肝細胞のHDL受容体（scavenger receptor (SR) type B1）を介して取り込まれる過程、さらに、HDL中のCEがコレステリルエステル転送蛋白（CETP）により、VLDL、IDL、LDLのアポB含有リポ蛋白へ転送され、アポB含有リポ蛋白が肝臓のLDL受容体などを介し肝臓に取り込まれる過程がある。このような代謝経路のいくつかのステップを修飾することでコレステロール逆転送を亢進させることが、新たな動脈硬化の治療法となりうる。

細胞内核内転写因子liver X receptor（LXR）はマクロファージにおいては、小腸、肝臓などの細胞内のコレステロールセンサーとして働き、コレステロール逆転送に関与する様々な分子の発現をもたらす。マクロファージでは、ATP-binding cassette（ABC）A1やABCG1の発現を誘導しHDLによるコレステロールの引き抜きを促進し、またCETPの転写を亢進させる。さらには小腸からのコレステロール吸収を阻害するなど、LXRは抗動脈硬化作用を有すると考えられる。すでに、動物実験においてはLXRアゴニストが動脈硬化を抑制することが報告されており、またSREBP-1cなどを介した脂肪酸合成による脂肪肝の発現や血中トリグリセリドの上昇をもたらさないような、組織・細胞に選択的なLXRアゴニストの開発も行われている。

HDLの観点からは、HDL自身を増加させる治療法が最も有用と考えられる。既存の高脂血症治療薬のうち、プロブコール以外の薬剤には、程度の差こそあれHDL増加作用がある。低HDL血症患者を対象としたVeterans Affairs Low HDL Cholesterol Intervention Trial（VA-HIT）では、フィブラート系薬剤ジェムフィブロジルによりHDLが平均6%上昇し、LDLには有意な変化はないものの冠動脈疾患の罹病率、死亡率が減少したことが示された。また、ニコチン酸およびスタチンにもHDL上昇作用があり、これらの併用はそれぞれのHDL増加効果を増強させる。さらに、血中HDL濃度に影響を与えるものとしてCETP濃度・活性があり、両者は逆相関する。このことより、CETP活性の阻害は抗動脈硬化作用を有すると考えられる。すでにCETP阻害薬の開発が行われ、動物実験では動脈硬化病変を抑制することが示されている[1]。ただし、CETPの阻害はコレステロール逆転送系を抑制するという反面もあり、現在、本邦において行われているCETP阻害薬の臨床治験第Ⅱ相の結果が待たれる。

一方、近年、新規リポ蛋白リパーゼであるendothelial lipase（EL）がHDLの血中濃度を規定する分子であることが明らかになった。本リパーゼは、HirataらおよびRaderらにより、同時期に別にクローニングされた分子であり、lipoprotein lipaseと44%、hepatic lipaseと41%の相同性を有し、血管内皮から産生され、おそらく産生された局所で作用し、種々の炎症性サイトカインにより発現を調節されているという特色をもつ[2]。

図1 EL過剰発現マウス（Tg）、EL欠損マウス（KO）、および野生型（WT）の血清脂質



生体内でのELの発現量は血清HDL値を規定する。

マウスにおいてアデノウイルスベクターを用いEL遺伝子を一過性に過剰発現させると、HDLがほぼゼロのレベルまで著明に減少したことから、ELがHDLの血中濃度を規定することが考えられるようになった。そしてその後、遺伝子操作マウスを用いた検討において、血中HDL濃度が、EL遺伝子欠損マウスのホモ接合体で約50%、ヘテロ接合体では25%、それぞれ増加し、逆にEL過剰発現マウスでは20%低下した[3]（**図1**）。そしてこの増減はHDL粒子の数の変化を反映していることが報告された。さらに、ヒトにおいてもELの遺伝子多型が血中HDL値に影響を与えることが明らかにされた。EL遺伝子欠損マウスとapo E遺伝子欠損マウスとの交配実験において、ELの欠損が動脈硬化を軽減することも報告された。ELの動脈硬化促進作用の機序は現在まだ十分には判明していないが、apo B含有リポ蛋白の異化を亢進させること、肝臓や腎でのapo A-Iのuptakeを亢進させること、また非酵素活性依存性にheparan sulfate proteoglycanを介して単球と内皮の接着を増強する作用などが関係すると考えられている。このような知見により、現在まだその活性、発現を抑制する薬剤は開発されていないが、ELも動脈硬化治療の標的分子になると考えられる。

コレステロール代謝の観点からの治療戦略に関しては、このほかにも、小腸粘膜で吸収したコレステロールをエステル化するACAT（acyl-CoA：cholesterol acyltransferase）、小腸でのミクロゾーム・トリグリセリド転送蛋白（MTP）、肝細胞のレムナント（apo E）受容体、そして既に述べた肝細胞のSR-B1やマクロファージからのコレステロール引き抜き受容体であるABCA1など多彩な分子が標的分子になり得ると考えられる[4]。

●3. メタボリックシンドロームの観点からの治療

一方、これから重要となってくると考えられるのが、メタボリックシンドロームの観点からの動脈硬化性疾患の治療である。メタボリックシンドロームの中核をなすのは肥満、耐糖能異常であり、高血圧、脂質代謝異常（高トリグリセリド血症、低HDL血症）などを合併しており、これらのいずれもが冠危険因子である。そして、CRP高値、PAI-1上昇など炎症や易血栓性なども合併しており、その機序としては種々の因子が関わっていると推定されるが、この病態の基盤に肥満とインスリン抵抗性が存在すると考える大きな流れができつつある。現在、臨床で用いられている薬剤のうち、フィブラート系薬剤およびチアゾリジン誘導体はそれぞれ、核内受容体PPARαとPPARγのリガンドとして働きインスリン抵抗性を改善する。またメトホルミンはAMPK（AMP-activated protein kinase）を介してインスリン抵抗性、ならびにメタボリックシンドロームの病態を改善する。

一方、最近特に注目を浴びている分子としてアディポネクチンがあげられる。アディポネクチンに関し

ては、既に本誌でも取り上げられているので詳細は割愛するが、脂肪組織により産生されるサイトカイン（アディポサイトカイン）であり、その血中濃度はインスリン抵抗性と強く相関し、2型糖尿病患者では血中アディポネクチン濃度が低下している。そして、アディポネクチン遺伝子のミスセンス変異を持つ者では血中アディポネクチン濃度の低下が生じ、非常に高頻度に冠動脈疾患を発症することが明らかにされ、さらに、冠動脈疾患患者において血中アディポネクチン濃度は独立した負の発症の規定因子であることも明らかになった[5]。そして、アディポネクチンの遺伝子欠損マウスにおいてバルーン障害後の内膜肥厚が増強し、さらにapo E遺伝子欠損マウスに対し尾静脈よりアデノウイルスを用いてアディポネクチンの遺伝子導入を行うと、血中アディポネクチン濃度の増加ならびに動脈硬化領域の軽減が認められたと報告されている。これらのことより、アディポネクチンが抗糖尿病分子であるだけでなく、抗動脈硬化分子として重要であることが明らかになった。そこで、アディポネクチンに焦点を当てた動脈硬化の治療戦略が考えられる。現在既に用いられている薬剤としてはチアゾリジン誘導体があげられる。本薬剤はPPARγリガンドであり、アディポネクチンプロモーターに働き、アディポネクチンを転写レベルで増加させ、血中アディポネクチン濃度を増加させる。PPARγリガンドによるアディポネクチンの転写調節には、オーファン核内受容体LRH-1が関与することも判明しており、今後、より選択的なアディポネクチン産生増強薬の開発が期待される。一方、二つのアイソフォームからなるアディポネクチン受容体も同定されており、高インスリン血症により同受容体がダウンレギュレーションされ、インスリン抵抗性がさらに増悪することも示された。アディポネクチン受容体をアップレギュレーションする薬剤も動脈硬化の治療薬として有望と思われる。

●4. 内皮障害・炎症の観点からの治療

動脈硬化を慢性炎症として捉える立場からは、その始まりとなる内皮細胞の障害・活性化を抑制する治療法が考えられる。この観点からの治療法の標的分子の中心となるのはNOであり、ACE阻害薬、アンジオテンシンタイプ1受容体拮抗薬、さらにはスタチンの作用機序の一部に内皮型NO合成酵素（eNOS）の活性・発現を増強させることがあげられている。一方、最近になり糖尿病や高脂血症においては、eNOS uncouplingと称される現象が生じ、eNOSがNOよりもむしろスーパーオキシドを産生することが明らかになった。この機序としては、eNOSがNO産生酵素としての作用を発揮する際に必須である補酵素、テトラビオプテリン（BH4）の内皮細胞内の濃度が、これらの病態下では低下していることがあげられる。スーパーオキシドの増加は動脈硬化の促進に働くわけであり、動脈硬化の薬物治療においては、やみくもにeNOS発現を増加させるのではなく、同時にBH4の細胞内濃度を増加させる戦略が必要である[6]。今後のNOに立脚した動脈硬化治療法としては、BH4の発現増加、活性増加を誘導する薬剤が必要と考えられ、動物実験では、BH4合成の律速酵素であるGTPCH-1の遺伝子導入や、あるいはBH4そのものの慢性投与により、動脈硬化病変の進展が抑制されることが示されている。ヒトにおいては合成BH4の投与もありうるが、その場合血管への移行性、大量投与に伴う副作用など解決すべき問題はまだ多い。一方GTPCH-1の活性、発現の増加をめざした治療法に関しても、いまのところスタチンがBH4の発現を誘導することが示されている以外は報告は認められていない。

●5. 免疫の観点からの治療

最後に、これまであまり取り上げられていないが、将来発展していく可能性のある動脈硬化性疾患の治療法として、免疫療法が考えられる[7]。生体の免疫機序には自然免疫と獲得免疫があり、近年自然免疫における反応開始分子として炎症細胞の細胞膜受容体Toll-like receptor（TLRs）の存在が明らかになり、動脈硬化巣においてTLRsが発現していることが示された。しかしながら、TLRsの動脈硬化における意義はまだ明らかとはいえず、TLRの観点からの治療法もまだ報告されていない。一方、獲得免疫は、侵入した異物を抗原提示細胞が認識し、それに続くTリンパ球や

Bリンパ球などが関与した一連の免疫反応をさす。Tリンパ球は動脈硬化巣にも浸潤しており、その役割が注目されている。特にCD4陽性のTリンパ球はTh1とTh2のサブセットに分けられ、前者はインターフェロンγ、インターロイキン12などを産生し細胞性免疫の活性化に関わり、後者はインターロイキン4やインターロイキン10などを産生し、おもにBリンパ球を介した液性免疫の活性化に関与する。主としてマウスを用いた検討において、Th1は動脈硬化の促進に、Th2は動脈硬化に防御的に働くことが示され、Th2を誘導するような操作により動脈硬化の進展を抑制できることが明らかになっている。我々もapo E遺伝子欠損マウスにIL10遺伝子を骨格筋内に遺伝子導入することで、CD4陽性T細胞がTh2優位となり、動脈硬化病変が抑制されることを見出している[8]。また、Bリンパ球による液性免疫の役割も注目されてきており、apo E遺伝子欠損マウスに対し、非特異的免疫グロブリンを投与することで動脈硬化が軽減することも報告されている。

また、動脈硬化に関係する種々の分子に対する抗体を投与することも、実験レベルでは動脈硬化を抑制することが示されている。しかしながら臨床における合成抗体の投与は、それ自身の抗体を誘導し、長期的な効果は期待できない。そこで近年考えられているのが自己抗体を誘導するワクチンの手法である。動脈硬化の初期病変形成には、何らかの抗原に対しての免疫反応が重要との認識がされつつあり、その抗原として注目されているのが酸化LDLおよびHSPである。酸化LDLはマクロファージなどの泡沫化に必須であり、HSPは細胞障害ストレスに対する生体防御作用として発現していると解釈されるが、一方においてこれらの蛋白はTLR4のリガンドとして働く。この3～4年の間に相次いでこれらに対しての抗体産生を誘導することで動脈硬化が抑制できる可能性が示された[9]。すなわち、apo E遺伝子欠損マウスにおいて酸化LDLやHSPをワクチンとして投与し、免疫を誘導すると動脈硬化が抑制できることが報告された。酸化LDLに関しては、酸化LDLに特異的に結合し、マクロファージの泡沫化を抑制する抗体が同定され、EO抗体と命名された。さらにその中のEO6抗体が、肺炎球菌を初めとした細菌の表面に発現しているホスホリルコリンを認識する抗体、T15抗体と同一であることが判明した。以前より臨床において使用されている肺炎球菌ワクチンは、このT15抗体を介し、肺炎球菌からの感染を防止すると考えられている。そこで、肺炎球菌ワクチンをLDL受容体遺伝子欠損マウスに投与すると、動脈硬化病変が減少することが報告された。これらの知見をもとに、現在、米国では臨床において肺炎球菌ワクチンが動脈硬化を抑制するかどうかの検討が行われつつある。またCETPに対するワクチン療法の臨床研究も既に行われている。

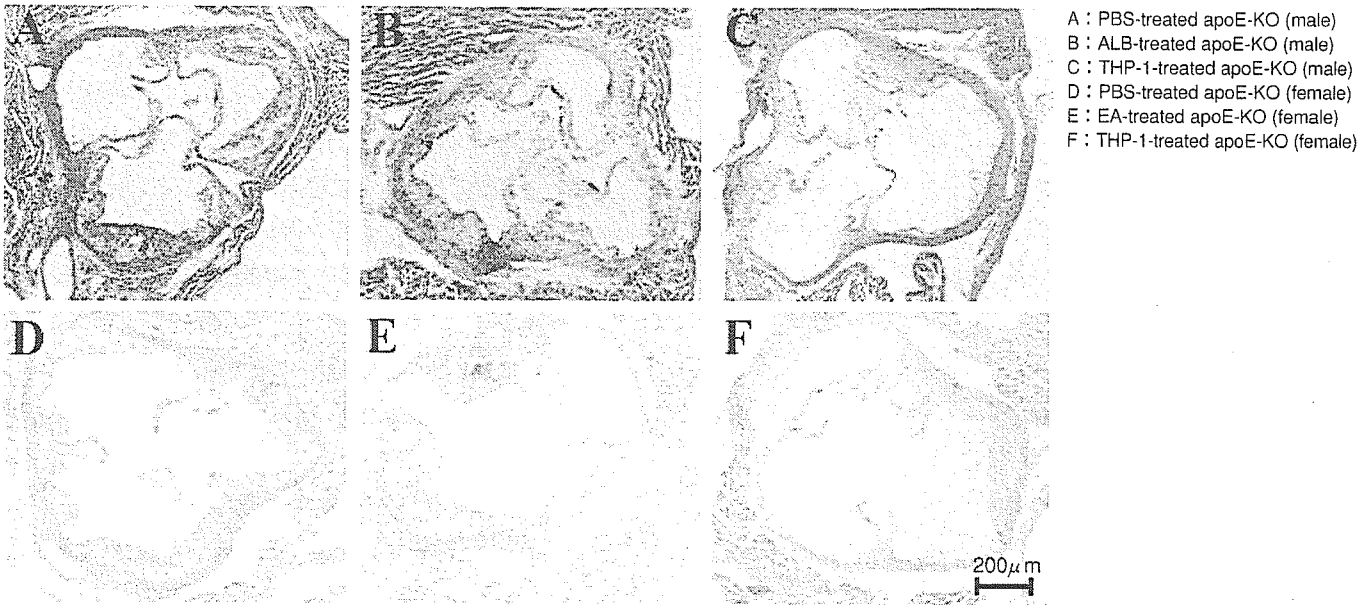
一方、我々は異種マクロファージをワクチンとして用いる方法を考案した。apo E遺伝子欠損マウスに異種であるヒトの単球・マクロファージをワクチンとして投与すると、おそらく交叉免疫により、マウスマクロファージ蛋白に対する抗体がマウスの血中に誘導された。そして、産生された抗体によりマクロファージ機能が修飾され、動脈硬化病変が抑制されていた(**図2**)。産生された抗体がターゲットとするマクロファージの分子の同定はできていないが、動脈硬化に対するワクチン療法の可能性を示すものと考えている。

既に述べたようにこれまでの免疫療法、ワクチン療法を用いた検討はほとんどマウスに限られており、この手法がヒトにも当てはまるかどうかという点や、ターゲットをどの分子にするかなど、今後検討すべきことは多いが、安価である利点などを考えると、ワクチン療法は一つの可能性として考えておくべき動脈硬化の将来の治療法と考える。

●6. おわりに

筆者が考えるこれからの動脈硬化の薬物治療の開発の方向性について述べた。はじめに述べたように、このような動脈硬化そのものの治療に加え、急性冠症候群など、個々の動脈硬化性疾患に対する治療戦略も重要であるが割愛した。高齢化、食生活の変化に伴い、これからもますます動脈硬化性疾患の重要性は増すと予想され、それに対し、筆者の触れなかった治療戦略も含め、様々な薬物治療が考えられていく

図2 異種単球・マクロファージを用いたワクチン療法による動脈硬化病変の減少
（apo E遺伝子欠損マウス）



であろう。ただ、注意しておかなければならないこととして、現在の薬物治療の基盤となっている基礎研究の多くが、遺伝子操作マウスなどを用いたマウスにおける研究が中心となっていることである。ヒトとマウスでは脂質代謝、免疫機構など多くが異なり、マウスのデータがそのままヒトには当てはまらない。マウスで得られたデータを鵜呑みにするのではなく、常にヒトではどうであるかを念頭に置くことにより、これからの動脈硬化の薬物治療が進歩することを期待したい。

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Stoichiometric Relationships Between Endothelial Tetrahydrobiopterin, Endothelial NO Synthase (eNOS) Activity, and eNOS Coupling in Vivo

Insights From Transgenic Mice With Endothelial-Targeted GTP Cyclohydrolase 1 and eNOS Overexpression

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Abstract—Endothelial dysfunction in vascular disease states is associated with reduced NO bioactivity and increased superoxide ($O_2^{\cdot-}$) production. Some data suggest that an important mechanism underlying endothelial dysfunction is endothelial NO synthase (eNOS) uncoupling, whereby eNOS generates $O_2^{\cdot-}$ rather than NO, possibly because of a mismatch between eNOS protein and its cofactor tetrahydrobiopterin (BH4). However, the mechanistic relationship between BH4 availability and eNOS coupling in vivo remains undefined because no studies have investigated the regulation of eNOS by BH4 in the absence of vascular disease states that cause pathological oxidative stress through multiple mechanisms. We investigated the stoichiometry of BH4–eNOS interactions in vivo by crossing endothelial-targeted eNOS transgenic (eNOS-Tg) mice with mice overexpressing endothelial GTP cyclohydrolase 1 (GCH-Tg), the rate-limiting enzyme in BH4 synthesis. eNOS protein was increased 8-fold in eNOS-Tg and eNOS/GCH-Tg mice compared with wild type. The ratio of eNOS dimer:monomer was significantly reduced in aortas from eNOS-Tg mice compared with wild-type mice but restored to normal in eNOS/GCH-Tg mice. NO synthesis was elevated by 2-fold in GCH-Tg and eNOS-Tg mice but by 4-fold in eNOS/GCH-Tg mice compared with wild type. Aortic BH4 levels were elevated in GCH-Tg and maintained in eNOS/GCH-Tg mice but depleted in eNOS-Tg mice compared with wild type. Aortic and cardiac $O_2^{\cdot-}$ production was significantly increased in eNOS-Tg mice compared with wild type but was normalized after NOS inhibition with *N*ω-nitro-L-arginine methyl ester hydrochloride (L-NAME), suggesting $O_2^{\cdot-}$ production by uncoupled eNOS. In contrast, in eNOS/GCH-Tg mice, $O_2^{\cdot-}$ production was similar to wild type, and L-NAME had no effect, indicating preserved eNOS coupling. These data indicate that eNOS coupling is directly related to eNOS–BH4 stoichiometry even in the absence of a vascular disease state. Endothelial BH4 availability is a pivotal regulator of eNOS activity and enzymatic coupling in vivo. (*Circ Res.* **2005;97:864-871.**)

Key Words: endothelial nitric oxide synthase ■ tetrahydrobiopterin ■ nitric oxide ■ superoxide

Nitric oxide (NO), produced by endothelial NO synthase (eNOS) in the vascular endothelium, is a critical signaling molecule in vascular homeostasis.[1] NO serves as an endothelium-derived relaxing factor, regulates vasomotor tone and blood pressure,[1,2] and has multiple antiatherogenic roles by inhibiting vascular smooth muscle cell proliferation, platelet aggregation, and leukocyte adhesion.[1] Loss of NO bioavailability is a key feature of endothelial dysfunction in vascular disease states such as hypertension, diabetes, and atherosclerosis. Furthermore, impaired NO-mediated endothelial function is an independent risk factor for cardiovascular disease.[3–5] Several factors contribute to loss of NO bioavailability, including reduced NO synthesis and NO scavenging by reactive oxygen species (ROS).[6] Under physiological conditions, there is a balance between endothelial NO and ROS production. However, vascular diseases are associated with increased ROS generation.[6] Several oxidase systems contribute to the increased oxidative stress, notably the NADPH oxidases.[7,8]

Increasing evidence suggests that eNOS itself can generate superoxide ($O_2^{\cdot-}$) under certain pathophysiological conditions.[9] Ozaki et al[10] reported recently that transgenic overexpression of eNOS in apolipoprotein E knockout mice paradoxically increases vascular $O_2^{\cdot-}$ production because of

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enzymatic uncoupling of increased eNOS protein levels. Recent data indicate that the pterin cofactor tetrahydrobiopterin (BH4) is a major determinant of whether eNOS produces NO or $O_2^{\cdot-}$.[11,12] When BH4 levels are insufficient, there is a shift toward the production of $O_2^{\cdot-}$ as electron transfer within the active site of eNOS becomes uncoupled from L-arginine oxidation, and molecular oxygen is instead reduced to form $O_2^{\cdot-}$.[11] $O_2^{\cdot-}$ generated by eNOS has been implicated in endothelial dysfunction associated with a number of vascular disease states, including diabetes, smoking, hypertension, and atherosclerosis,[10,12–16] and BH4 supplementation improves endothelium-dependent vasodilatation under these conditions.[16] However, the effects of systemic pharmacological BH4 supplementation in these studies may be mediated in part by nonspecific antioxidant properties of acute high-dose BH4,[17] which can increase NO bioavailability indirectly by reducing its scavenging by ROS.

Recent studies have focused on the potential role of BH4 oxidation, to dihydrobiopterin (BH2) and other biopterin species, in reducing BH4 bioavailability in preatherosclerotic disease states.[16–18] In particular, the interaction of BH4 with peroxynitrite (generated from the reaction between NO and $O_2^{\cdot-}$) rapidly oxidizes BH4 and can provoke eNOS uncoupling and endothelial dysfunction.[12,19–21] Indeed, eNOS uncoupling may exacerbate the process by contributing to BH4 oxidation. However, it is unclear whether eNOS uncoupling alone is sufficient to initiate BH4 oxidation and exacerbate eNOS uncoupling in vivo because all in vivo studies to date have evaluated BH4-dependent eNOS regulation in complex vascular disease states in which multiple inflammatory and redox pathways are implicated. Other previous studies of the role of BH4 in eNOS function have relied on purified recombinant proteins in reconstituted cell-free systems.[9,11,22,23]

Accordingly, we sought to investigate the importance of BH4 in regulating eNOS activity in vivo in healthy animals without vascular disease. We used a transgenic mouse model with endothelial-targeted overexpression of GTP cyclohydrolase 1 (GTPCH), the rate-limiting enzyme in BH4 synthesis, in which endothelial BH4 levels are specifically increased.[24] We crossed this transgenic mouse with a mouse overexpressing eNOS in the endothelium to generate mouse models with graded alterations in endothelial BH4 and eNOS levels to investigate the mechanistic relationships between BH4 and eNOS coupling in vivo.

Materials and Methods

Animals

All studies involving laboratory animals were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act 1986 (HMSO, UK). eNOS transgenic (eNOS-Tg) mice, in which bovine eNOS transgene overexpression is targeted to the vascular endothelium under the control of the murine preproendothelin-1 promoter in a C57BL/6 background, were produced as described previously.[25] GTPCH transgenic (GCH-Tg) mice, in which human GTPCH transgene overexpression is targeted to the endothelium under control of the murine Tie-2 promoter, were generated in a C57BL/6 background as described previously.[26] Heterozygote eNOS-Tg mice were mated with heterozygote GCH-Tg mice to produce experimental eNOS/GCH-Tg, eNOS-Tg, GCH-Tg, and wild-type littermates in a 1:1:1:1 ratio. Mice (between 13 and 20 weeks of age in all experiments) were housed in individually ventilated cages with 12-hour light/dark cycle and controlled temperature (20°C to 22°C) and fed normal chow and water ad libitum.

Western Blot Analysis

Lung samples ($n \geq 4$ per group) were homogenized on ice for 20 seconds in lysis buffer (50 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, 0.1% SDS, 0.5% deoxycholate, 1% Nonidet P-40) containing protease inhibitors (Complete; Boehringer Mannheim) and 1 mmol/L phenylmethylsulfonyl fluoride. Protein lysates (8 μg) were resolved using SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were incubated with a 1:2000 dilution of mouse anti-eNOS monoclonal antibody (Transduction Laboratories), which recognizes murine and bovine eNOS, followed by a 1:2500 dilution of rabbit anti-mouse horseradish peroxidase–conjugated secondary antibody (Promega). Protein bands were visualized by chemiluminescence. To investigate the ratio of eNOS homodimer to monomer, Western blots were performed as above using nonboiled aortic lysates and low-temperature SDS-PAGE as described previously.[27]

Primary Cultures of Murine Lung Endothelial Cells

Lungs were harvested into culture medium (35% DMEM, 35% Ham's F-10 nutrient mixture, 20% FBS, 2 mmol/L L-glutamine, 100 U/100 μg/mL penicillin–streptomycin, 100 μg/mL heparin, and 50 μg/mL endothelial mitogen [Biogenesis]), cut into 1- to 2-mm pieces and digested using 0.1% collagenase type I for 1 hour at 37°C. The lung digest was passed through a 100-μm cell strainer. Cells were centrifuged, resuspended in culture medium, and plated onto 0.1% gelatin-coated cover slips. Cultures were maintained at 37°C in humidified 5% CO_2/95% air atmosphere for 72 hours before fixation with 4% paraformaldehyde.

Immunocytochemistry

Fixed cultures were permeabilized with PBS containing 0.5% Triton X-100, and nonspecific staining was reduced by blocking with 10% normal goat serum. Cultures were incubated with a polyclonal rabbit anti-eNOS primary antibody (Transduction Laboratories) followed by goat anti-rabbit secondary antibody (Alexa Fluor 488; Molecular Probes). Cells were mounted with cover slips using Vectashield containing propidium iodide (Vector Laboratories) and imaged using a Bio-Rad MRC-1024 laser-scanning confocal microscope.

Measurement of Biopterins and Neopterin

Biopterins, such as BH4, BH2 and biopterin, and neopterin were measured in aortic homogenates by high-performance liquid chromatography (HPLC) analysis after iodine oxidation in acidic or alkaline conditions as described previously.[24,28] In brief, thoracic aortas ($n=6$ to 8 per group) were homogenized for 20 seconds in ice-cold extract buffer (50 mmol/L Tris-HCl, pH 7.4, 1 mmol/L dithiothreitol, and 1 mmol/L EDTA) containing 0.1 μmol/L neopterin as an internal recovery standard. Samples were deproteinated before undergoing oxidation with 1% iodine/2% potassium iodide under either acidic or basic conditions. Biopterin content was assessed using HPLC in 5% methanol/95% water using an ACE 5 C18 column (ACT) and fluorescence detection (350 nm excitation and 450 nm emission). BH4 concentration was calculated as picomoles per milligram of protein by subtracting BH2 and biopterin from total biopterin content.

Arginine-to-Citrulline Conversion

NOS enzymatic activity, and indirectly NO synthesis, was measured by the conversion of ^{14}C L-arginine to ^{14}C L-citrulline in fresh intact aorta ($n=5$ to 8 per group) and lung homogenate ($n=6$ per group) as described previously.[24,29] The integrals of citrulline peaks were expressed as a proportion of total ^{14}C counts for each sample.

Electron Paramagnetic Resonance Spectroscopy

Electron paramagnetic resonance (EPR) spectroscopy was used to quantify vascular NO production according to previously described and validated methods.[30] In brief, freshly harvested aortas (n=8 to 11 per group) were stimulated with calcium ionophore (A23187; 1 μmol/L) in 100 μL Krebs–HEPES buffer, then incubated with colloid iron (II) diethyldithiocarbamate [$Fe(DETC)_2$] (285 μmol/L) at 37°C for 90 minutes. After incubation, aortas were snap-frozen in a column of Krebs–HEPES buffer in liquid nitrogen, and EPR spectra were obtained using an X-band EPR spectrometer (Miniscope MS 200; Magnettech). Signals were quantified by measuring the total amplitude, after correction of baseline, and after subtracting background signals from incubation with colloid $Fe(DETC)_2$ alone.

Lucigenin-Enhanced Chemiluminescence Detection of Superoxide in Heart Lysates

Basal O_2^- production was measured in left ventricular (LV) homogenates (n=7 to 10 per group) using the technique of lucigenin (5 μmol/L) chemiluminescence according to methods described previously.[14,31] In brief, hearts were flushed with ice-cold Krebs–HEPES buffer, the LV excised, and snap-frozen in liquid nitrogen. Samples were homogenized in Kreb–HEPES buffer containing protease inhibitors (Complete; Boehringer Mannheim) at pH 7.4. Chemiluminescence was measured in a FB12 luminometer (Berthold Detection Systems) at 37°C. Chemiluminescence of 200 μg LV protein was recorded every minute for 8 minutes. The NOS inhibitor *N*ω-nitro-L-arginine methyl ester hydrochloride (L-NAME; 1 mmol/L) was subsequently added and chemiluminescence recorded for an additional 5 minutes. Background readings were subtracted from sample readings and results expressed as counts per second.

Lucigenin-Enhanced Chemiluminescence Detection of Superoxide in Intact Aorta

Basal O_2^- production was measured in intact aorta (n=8 to 12 per group) according to methods described previously.[14,32] In brief, freshly cleaned and harvested thoracic aortas were opened longitudinally, cut into 2, and transferred to ice-cold Krebs–HEPES buffer. Vessels were equilibrated in Krebs–HEPES buffer gassed with 95% oxygen/5% carbon dioxide for 30 minutes at 37°C, with one half of each vessel being incubated in the presence of L-NAME (1 mmol/L). Lucigenin (20 μmol/L) chemiluminescence was then recorded every minute for 10 minutes as above. Background readings were subtracted from sample readings and results expressed as counts per second per milligram dry weight of aorta.

Oxidative Fluorescent Microtopography

O_2^- production in tissue sections of mouse aorta (n=5 to 7 per group) was detected using the fluorescent probe dihydroethidium (DHE), as described previously.[14,24,33] Fresh segments of thoracic aorta were frozen in optimal cutting temperature compound. Cryosections (30 μm) were incubated with Krebs–HEPES buffer with or without L-NAME (1 mmol/L; to inhibit eNOS) for 30 minutes at 37°C, then for an additional 5 minutes with DHE (2 μmol/L; Molecular Probes). Images were obtained using a Bio-Rad laser-scanning confocal microscope, equipped with a krypton/argon laser, using identical acquisition settings for each section. DHE fluorescence was quantified by automated image analysis using Image-Pro Plus software (Media Cybernetics). DHE fluorescence from high power (×60) images was measured only on the luminal side of the internal elastic lamina to quantify endothelial cell fluorescence. For each vessel, mean fluorescence was calculated from 4 separate high-power fields taken in each quadrant of the vessel to produce n=1, and all experiments were performed in a batch design.

Statistical Analysis

One-way ANOVA tests were used to compare data sets, with appropriate post hoc correction for multiple comparisons. $P<0.05$ was considered significant. Data are expressed as means and SEM.

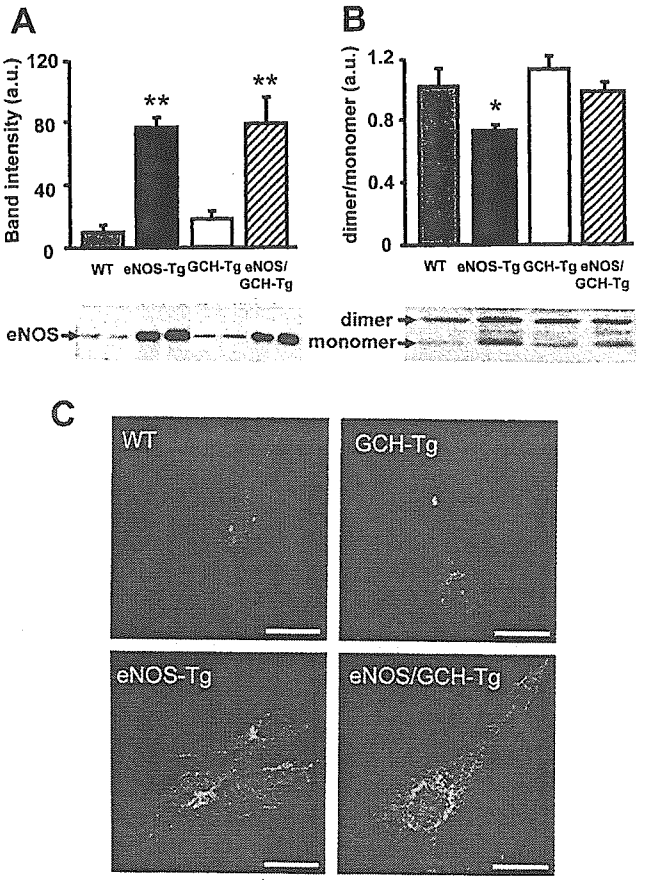

Figure 1. Immunoblotting with a murine anti-eNOS monoclonal antibody to detect native and transgenic eNOS monomer protein in boiled lung lysates (A) and eNOS dimer:monomer protein bands in aortic lysates from wild-type (WT), eNOS-Tg, GCH-Tg, and eNOS/GCH-Tg mice (B); n=4 animals per group; $^*P<0.05$ and $^{**}P<0.001$ compared with WT. a.u. indicates arbitrary units. C, Immunofluorescent detection of eNOS (green), counterstained with propidium iodide (red), in primary endothelial cells cultured from WT, eNOS-Tg, GCH-Tg, and eNOS/GCH-Tg mice. Bar=20 μm.

Results

eNOS Protein Levels and Subcellular Localization

Western blot analysis confirmed that eNOS protein levels were elevated 8-fold in eNOS-Tg compared with wild-type animals ($P<0.001$; Figure 1A). Overexpression of endothelial GTPCH, the rate-limiting enzyme in BH4 synthesis, in GCH-Tg mice did not significantly alter eNOS protein levels compared with wild type. However, as for eNOS-Tg mice, eNOS protein levels were elevated 8-fold in double-transgenic eNOS/GCH-Tg mice.

We used low-temperature SDS-PAGE and immunoblotting to investigate eNOS homodimerization and the ratio of eNOS dimer to monomer in aortas. In eNOS-Tg aortas, eNOS dimer:monomer was significantly depleted compared with wild type ($P<0.05$) but unchanged in GCH-Tg mice (Figure 1B). Importantly, the reduced eNOS dimer:monomer ratio in the eNOS-Tg group was restored to wild-type levels in double- transgenic eNOS/GCH-Tg mice.

We investigated the subcellular localization of eNOS in primary cultures of lung endothelial cells using immunocytochemistry. eNOS appeared to be localized mainly to plasma

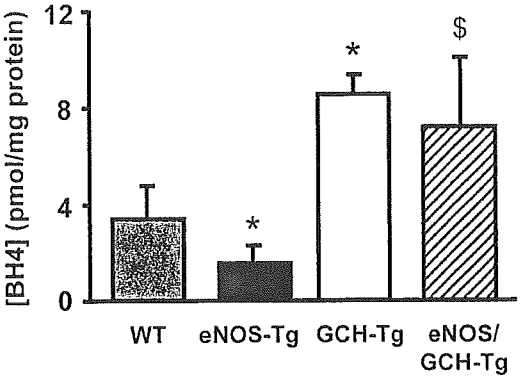

Figure 2. BH4 levels in aortas from wild-type (WT), eNOS-Tg, GCH-Tg, and eNOS/GCH-Tg mice. *$P<0.05$ compared with WT and \$$P<0.05$ compared with eNOS-Tg; n=6 to 8 animals per group.

membranes and the Golgi apparatus in endothelial cells from all 4 groups (Figure 1C). However, in accordance with the immunoblotting data, the intensity of eNOS immunostaining, unchanged in GCH-Tg mice, was markedly increased in endothelial cells from eNOS-Tg and eNOS/GCH-Tg animals compared with wild type.

Aortic BH4 Levels

We next measured vascular BH4 levels in homogenates of snap-frozen aorta using iodine oxidation and HPLC. Surprisingly, BH4 levels were significantly depleted in eNOS-Tg mice compared with wild type, suggesting oxidative degradation of BH4 ($P<0.05$; Figure 2). We then sought to confirm that increased endothelial GTPCH expression led to increased BH4 levels in aortic homogenates of GCH-Tg and eNOS/GCH-Tg mice. As reported previously,[24] aortic BH4 levels were significantly elevated by >2-fold in GCH-Tg mice compared with wild type ($P<0.05$). Importantly, aortic BH4 levels were also elevated in eNOS/GCH-Tg mice and were not significantly different between GCH-Tg and eNOS/GCH-Tg mice.

eNOS Enzymatic Activity and NO Production

To determine the relationship between eNOS protein levels and eNOS enzymatic activity, we measured conversion of ^{14}C L-arginine to ^{14}C L-citrulline by eNOS in intact aorta using HPLC with online scintillation detection. Citrulline production was increased only 2-fold in eNOS-Tg aortas compared with wild type ($P<0.05$; Figure 3A and 3B), despite eNOS protein levels being elevated 8-fold in these animals. Indeed, the ratio of eNOS enzymatic activity to eNOS protein was 0.6 in eNOS-Tg mice compared with 2.0 in wild-type animals. A similar pattern of results was obtained when using lung tissue lysates (Figure 3C). To further investigate the stoichiometric relationship between eNOS and endothelial BH4 in vivo and to determine whether increasing endothelial BH4 in eNOS-Tg mice could augment eNOS enzymatic activity, we next measured eNOS enzymatic activity in GCH-Tg and eNOS/GCH-Tg mice. NOS activity was increased 2-fold in GCH-Tg aorta and lung compared with wild type ($P<0.05$; Figure 3A through 3C). Indeed, eNOS enzymatic activity was similar in GCH-Tg and eNOS-Tg mice despite eNOS protein

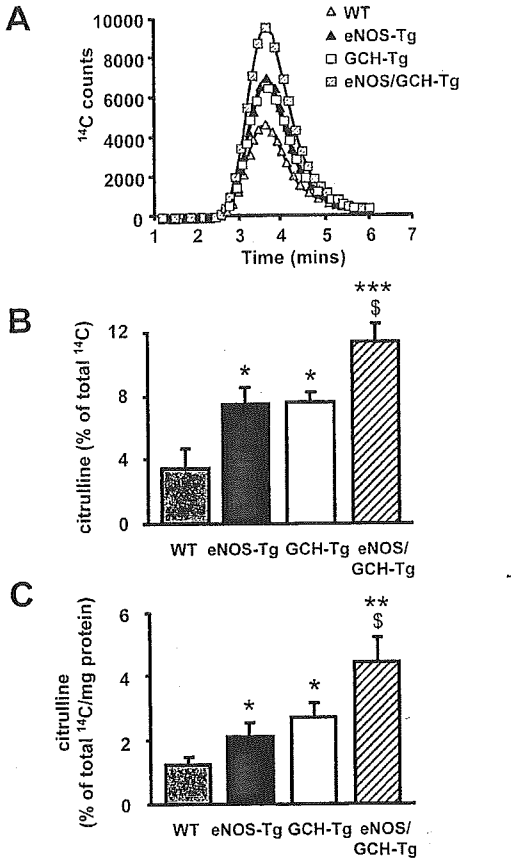

Figure 3. A, Representative HPLC chromatograms showing ^{14}C citrulline peaks for wild-type (WT; gray triangles), eNOS-Tg (black triangles), GCH-Tg (white squares), and eNOS/GCH-Tg (hatched squares) mouse aortas. Graphs show percentage ^{14}C citrulline conversion from ^{14}C arginine as a measure of eNOS activity measured in total fresh intact aorta (B) and lung tissue lysates (C); n=5 to 8 animals per group; *$P<0.05$, **$P<0.01$, and ***$P<0.001$ compared with WT; and \$$P<0.05$ compared with eNOS-Tg.

levels being considerably higher in eNOS-Tg animals. Critically, eNOS enzymatic activity was further elevated in eNOS/GCH-Tg mice compared with eNOS-Tg animals ($P<0.05$): augmented levels of endothelial BH4 in eNOS/GCH-Tg mice resulted in an ≈4-fold increase in eNOS enzymatic activity in aorta and lung compared with wild-type mice ($P<0.01$). These data suggest that eNOS activity is exquisitely dependent on endothelial BH4 levels even in the absence of vascular disease.

In complementary experiments, we used Fe-DETC EPR to directly measure NO bioavailability in mouse aortas. In accordance with measures of enzymatic activity, net NO levels were increased ≈2-fold in eNOS-Tg aortas compared with wild type (Figure 4). These results demonstrate that there was a striking discordance between eNOS protein levels, eNOS enzymatic activity, and NO production in eNOS-Tg mice. We then determined the effects of increased endothelial BH4 using the GCH-Tg and eNOS/GCH-Tg mice and observed a similar pattern of results as for NOS enzymatic activity. Aortic NO bioavailability was elevated almost 2-fold in GCH-Tg mice compared with wild type and not significantly different from eNOS-Tg mice (Figure 4). Criti-

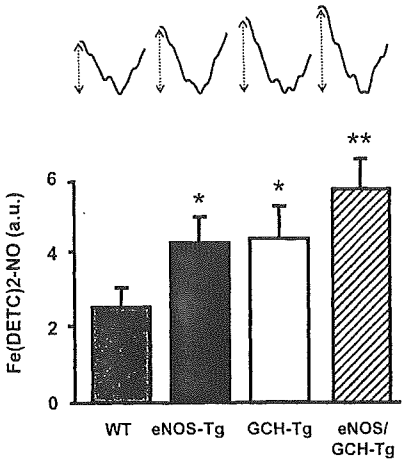

Figure 4. Net NO levels in intact aorta measured using Fe-DETC EPR. Graph shows mean quantitative data with corresponding representative EPR spectra showing the characteristic peaks associated with the Fe-DETC signal above. n=8 to 11 animals per group; *P<0.05 and **P<0.01 compared with wild type (WT). a.u. indicates arbitrary units.

cally, net NO bioavailability was further elevated (≈3-fold compared with wild type) in eNOS/GCH-Tg mice.

eNOS Uncoupling: Effect of eNOS and GTPCH Overexpression In Vivo

To investigate whether eNOS uncoupling results from discordance between eNOS and BH4, we measured $O_2^{\cdot-}$ production and, more specifically, eNOS-derived $O_2^{\cdot-}$ production using the NOS inhibitor L-NAME. We first measured $O_2^{\cdot-}$ production in tissue lysates using lucigenin chemiluminescence. Chemiluminescence was increased 2-fold in eNOS-Tg mice compared with wild-type animals (P<0.05; Figure 5A) but was unchanged in GCH-Tg mice. Critically, $O_2^{\cdot-}$ production was restored in eNOS/GCH-Tg mice. The proportion of $O_2^{\cdot-}$ production attributable to uncoupled NOS, assessed by quantifying L-NAME–inhibitable chemiluminescence, was significantly increased in eNOS-Tg lysates compared with wild type, indicating increased NOS uncoupling (P<0.05; Figure 5B). L-NAME–inhibitable chemiluminescence was unchanged in GCH-Tg mice. The presence of th GTPCH transgene in eNOS/GCH-Tg mice restored the er hanced L-NAME–inhibitable chemiluminescence of th eNOS-Tg group back to wild-type levels. We also investi gated $O_2^{\cdot-}$ production in intact aorta under basal condition and after incubation with L-NAME using lucigenin chemilu minescence and saw a similar pattern of results. Basa chemiluminescence was significantly increased in eNOS-T aortas compared with wild type (P<0.05; Table). Impor tantly, basal $O_2^{\cdot-}$ production in GCH-Tg and eNOS/GCH-T aortas was similar to wild type. Incubation of aortas wit L-NAME caused a significant reduction in the $O_2^{\cdot-}$ signal i eNOS-Tg mice (P<0.05), indicating NOS uncoupling. How ever, L-NAME had little effect in wild-type, GCH-Tg, an eNOS/GCH-Tg aortas, suggesting that NOS coupling i preserved in these mice. Together, these observations sugges that in eNOS-Tg mice elevated $O_2^{\cdot-}$ production is at leas partly attributable to uncoupled NOS, likely resulting fron discordance between eNOS protein and endothelial BH4 because NOS coupling is preserved by increasing endothelia BH4 in association with elevated eNOS levels in eNOS GCH-Tg animals.

Lucigenin Chemiluminescence to Measure $O_2^{\cdot-}$ Production in Intact Aortas Incubated for 30 Minutes at 37°C in the Presenc or Absence of L-NAME (1 mmol/L)

	Wild Type	eNOS-Tg	GCH-Tg	eNOS/GCH-T
Basal, RLU/s/mg	39.5±4.5	59.7±6.6*	52.8±5.8	46.2±4 7
+ L-NAME, RLU/s/mg	42.5±10.1	39.2±4.8†	54.0±13.2	49 3±11.2

Results are expressed as counts per second per milligram of dry weight aorta.

*P<0.05compared with wild type; †P<0.05 compared with baselir (without L-NAME).

RLU indicates relative light units.

To investigate $O_2^{\cdot-}$ production specifically from the aorti endothelium, we quantified endothelial DHE fluorescenc using oxidative confocal microtopography. Endothelial DHI fluorescence was increased 2-fold in eNOS-Tg mice com pared with wild-type and GCH-Tg mice (Figure 6). Impor

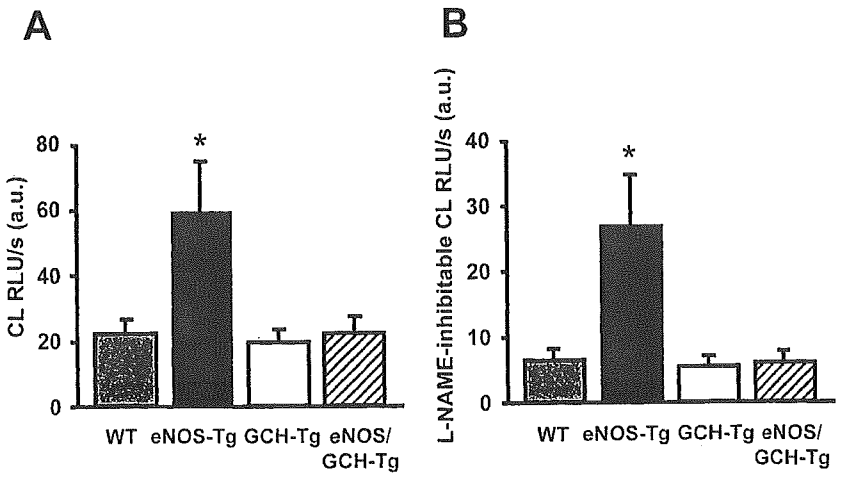

Figure 5. Lucigenin (5μmol/L) chemiluminescence (CL) in cardiac tissue lysates from wild-type (WT), eNOS-Tg, GCH-Tg, and eNOS/GCH-Tg mice to measure basal $O_2^{\cdot-}$ production (A) and L-NAME (1 mmol/L)–inhibitable $O_2^{\cdot-}$ production (B) as a marker of NOS uncoupling. *P<0.05 compared with WT; n=7 to 10 animals per group. RLU indicates relative light units, a.u., arbitrary units.

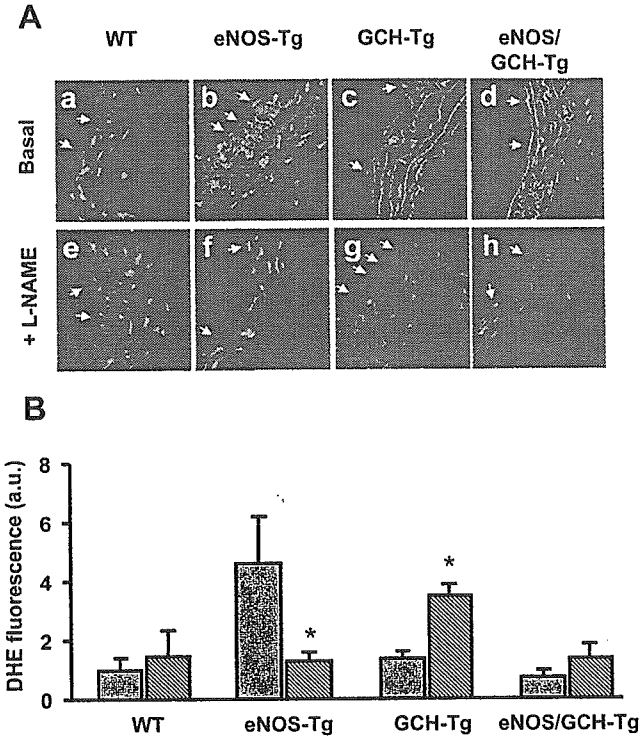

Figure 6. DHE staining, to measure in situ $O_2^{\cdot-}$ production, in aortic sections. A, Representative aortic sections (×60) showing red endothelial cells (arrows) from wild-type (WT; a and e), eNOS-Tg (b and f), GCH-Tg (c and g), and eNOS/GCH-Tg (d and h) mice in the presence (e through h) and absence (a through d) of L-NAME (1 mmol/L). B, Quantified specific endothelial DHE fluorescence is expressed for sections in the presence (hatched bars) and absence (gray bars) of L-NAME in arbitrary units (a.u.) for each group. *$P<0.05$ comparing sections in the presence or absence of L-NAME; n=5 to 7 animals per group.

tantly, endothelial fluorescence was restored to wild-type levels in eNOS/GCH-Tg mice. Fluorescence from the other layers of the vessel wall was not significantly different between groups. Incubation with L-NAME had little effect in wild-type aortas but reversed the elevated DHE signal in eNOS-Tg endothelium back to wild-type levels, again indicating that the source of $O_2^{\cdot-}$ was likely uncoupled eNOS. In contrast, L-NAME significantly increased the endothelial $O_2^{\cdot-}$ signal in GCH-Tg mice, indicating that in these aortas, eNOS was predominantly coupled and producing NO. Critically, as in wild-type aortas, NOS inhibition with L-NAME had little effect in eNOS/GCH-Tg mice, indicating restored eNOS coupling compared with eNOS-Tg animals. In accordance with the data for $O_2^{\cdot-}$ production measured by chemiluminescence, these results suggest that increased eNOS uncoupling in eNOS-Tg aortas increases eNOS-derived $O_2^{\cdot-}$, but that eNOS coupling is, at least in part, preserved by increased endothelial BH4 synthesis in eNOS/GCH-Tg mice.

Discussion

In this study, we describe a new double-transgenic mouse model in which endothelial-targeted overexpression of GTPCH leads to increased endothelial BH4 levels in mice with endothelial-targeted eNOS overexpression. We used this model to investigate the role of BH4 in the regulation of eNOS coupling in vivo, specifically in the absence of pathological oxidative stress associated with vascular disease states.[6] The major findings in this study are as follows. First, eNOS protein levels are markedly elevated in eNOS-Tg and eNOS/GCH-Tg mice but not in GCH-Tg animals, although the proportion of eNOS dimer to monomer is depleted only in eNOS-Tg aortas. Second, endothelial-specific overexpression of GTPCH is sufficient to increase vascular BH4 levels in GCH-Tg and in eNOS/GCH-Tg aortas, whereas BH4 levels are depleted in eNOS-Tg aortas. Third, this increase in BH4 is sufficient to augment vascular eNOS enzymatic activity even in GCH-Tg mice, which have unchanged eNOS protein levels. Indeed, eNOS activity is similar between GCH-Tg and eNOS-Tg mice despite eNOS-Tg mice having 8-fold more eNOS protein. Importantly, the increase in endothelial BH4 in eNOS/GCH-Tg mice further enhances eNOS activity and NO bioavailability compared with eNOS-Tg mice. Fourth, the discordance between endothelial BH4 and eNOS protein in eNOS-Tg mice results in uncoupled eNOS and increased NOS-derived $O_2^{\cdot-}$ production in tissue lysates and intact aorta. However, increased vascular BH4 in eNOS/GCH-Tg mice is sufficient, at least in part, to restore eNOS coupling, increase NO production, and reduce eNOS-dependent $O_2^{\cdot-}$ production.

These findings provide important insights into the role of endothelial BH4 synthesis in regulating eNOS activity and eNOS coupling even in the absence of vascular oxidative stress. Previous studies have reported that endothelial dysfunction in vascular diseases, such as hypertension,[12] diabetes,[24] and atherosclerosis,[26] is associated with increased $O_2^{\cdot-}$ production deriving principally from NADPH oxidases.[7,8] Landmesser et al[12] demonstrated that the increase in NADPH oxidase–derived $O_2^{\cdot-}$ in deoxycorticosterone acetate–salt hypertensive mice led to enhanced oxidation of BH4, resulting in eNOS uncoupling, increased eNOS-derived $O_2^{\cdot-}$ production, and reduced NO formation, thereby exacerbating oxidative stress. Oral supplementation with BH4, or a reduction in NADPH oxidase activity (using p47phox−/− mice), reversed eNOS uncoupling. However, the mechanistic relationship between eNOS and its cofactor BH4 has not been investigated in vivo in the absence of pathological oxidative stress. We now show that a stoichiometric discordance between eNOS protein and BH4 levels is alone sufficient to cause eNOS uncoupling, and that eNOS uncoupling in the absence of vascular disease is sufficient to deplete BH4 levels by oxidation. Laursen et al[19] demonstrated that peroxynitrite may be the principal ROS involved in oxidation of BH4.

NO, constitutively produced by eNOS in the vascular endothelium, is a potent vasodilator and exerts numerous vasoprotective antiatherogenic effects. Reduced NO bioactivity is an early feature of a number of vascular diseases, including atherosclerosis.[5] Short-term in vivo gene transfer of eNOS or neuronal NOS can improve NO-mediated vascular relaxation in atherosclerotic arteries.[34] However, previous studies investigating the possible vasoprotective effects of chronic eNOS overexpression in eNOS-Tg mice have yielded conflicting results. Kawashima et al[35] demonstrated reduced lesion formation after carotid artery ligation in eNOS-Tg mice. In contrast, Ozaki et al,[10] using the same strain of

Possible Role of Brain-Derived Neurotrophic Factor in the Pathogenesis of Coronary Artery Disease

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Background—The neurotrophin (NT) family, including nerve growth factor NT-3 and brain-derived neurotrophic factor (BDNF), has a critical role in the survival, growth, maintenance, and death of central and peripheral neurons. NTs and their receptors are expressed in atherosclerotic lesions; however, their significance in cardiovascular disease remains unclear.

Methods and Results—To clarify the role of NTs in the pathogenesis of coronary artery disease, NT plasma levels in the aorta, coronary sinus, and peripheral veins of patients with unstable angina (n=38), stable effort angina (n=45), and non–coronary artery disease (n=24) were examined. In addition, regional expression of BDNF in coronary arteries was examined in autopsy cases and patients with angina pectoris by directional coronary atherectomy. The difference in BDNF levels, but not NT-3, between the coronary sinus and aorta was significantly greater in the unstable angina group compared with the stable effort angina and non–coronary artery disease groups. Immunohistochemical investigations demonstrated BDNF expression in the atheromatous intima and adventitia in atherosclerotic coronary arteries. BDNF expression was enhanced in macrophages and smooth muscle cells in atherosclerotic coronary arteries. Stimulation with recombinant BDNF significantly enhanced NAD(P)H oxidase activity and the generation of reactive oxygen species in cultured human coronary artery smooth muscle cells.

Conclusions—BDNF has an important role in atherogenesis and plaque instability via the activation of NAD(P)H oxidase. (***Circulation.* 2005;112:2114-2120.**)

Key Words: circulation ■ coronary disease ■ free radicals ■ nervous system ■ stress

On January 17, 1995, the great Hanshin-Awaji earthquake hit Kobe, Japan, killing 6433 people. Thereafter, there was an increase in mortality from cardiac disease.[1] Chronic psychological stress appears to have an important role in cardiovascular diseases after traumatic events such as a major earthquake.[2] Psychological factors such as depression and acute and chronic stress are potent risk factors for coronary artery disease (CAD).[2] The precise mechanisms by which psychological factors cause cardiovascular disease, however, remain to be determined. Under psychological stress, the hypothalamus-pituitary-adrenal axis and sympathetic nerve system are activated, and a wide range of neurohumoral factors are dynamically regulated, including neurotrophins (NTs).

NTs form a family of dimeric polypeptides, which include nerve growth factor, brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5 in humans.[3–5] NTs have critical roles in the survival, growth, maintenance, and death of central and peripheral neurons.[5,6] Under psychological stress, the secretion of NTs from the hypothalamus, pituitary gland, and central and peripheral nerves is markedly altered.[7] The biological activities of NTs are mediated via the specific high-affinity receptors trkA, trkB, and trkC and the low-affinity NT receptor p75.[8,9] NTs and their receptors are expressed in nonneuronal tissues and various cell types such as developing heart,[10] spleen,[11] atherosclerotic vessels,[12] macrophages,[13] lymphocytes,[14] endothelial cells,[15] and vascular smooth muscle cells,[12] suggesting that NTs have diverse roles even in nonneural organs. The significance of NTs in cardiovascular disease remains to be elucidated.

Acute coronary syndrome occurs as a consequence of coronary plaque rupture and superimposed thrombus. Reactive oxygen species derived from NAD(P)H oxidase have a critical role in the pathogenesis of CAD and plaque instabil-

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TABLE 1. Patient Characteristics

	Non-CAD (n=24)	SAP (n=45)	UAP (n=38)	P
Age, mean (range), y	62 (43–78)	65 (46–85)	66 (53–78)	0.36
Male sex, %	63	67	66	0.94
Degree of coronary stenosis, %		88.6 (74.8–94.3)	91.2 (74.6–95.0)	0.63
Ejection fraction, %	63 (51–77)	55 (40–69)	60 (41–70)	0.52
Hypertension, n (%)	12 (50)	32 (71)	20 (53)	0.13
Diabetes, n (%)	10 (42)	22 (49)	19 (50)	0.80
Hyperlipidemia, n (%)	9 (38)	21 (47)	20 (53)	0.62
Smoking, n (%)	10 (42)	19 (42)	17 (45)	0.96
Obesity, n (%)	9 (38)	20 (44)	15 (40)	0.83

Degree of coronary stenosis and ejection fraction are given as medians and interquartile ranges. Numbers of diseased vessels are given as mean and SEM.

ity.[16] BDNF induces oxidative stress via the activation of this oxidase system in cortical cells.[17] The localization of NTs in the cardiovascular system and their potent biological activities suggest a possible role for these neurotrophic molecules in the pathogenesis of cardiovascular disease, including acute coronary syndrome. To clarify the significance of NTs in the pathogenesis of CAD, we examined NT plasma levels in the coronary circulation of patients with angina pectoris and non-CAD and their regional expression in coronary arteries obtained from autopsied cases and coronary specimens obtained during directional coronary atherectomy (DCA). Furthermore, we examined the pro-oxidative effects of NTs on cultured vascular cells.

Methods

Patient Groups

Patients who underwent diagnostic coronary angiography and patients with angina in whom significant stenosis of the left coronary arteries was documented were enrolled. Subjects were divided into unstable angina (UAP), stable effort angina (SAP), and non-CAD groups. Table 1 shows the clinical characteristics of the 3 groups. The UAP group consisted of 38 patients who had anginal episodes at rest or angina during a mild degree of effort within 48 hours of the study without a significant increase in creatine phosphokinase levels. Patients were classified IB (n=14), IIB (n=12), and IIIB (n=12) according to Braunwald's criteria. The SAP group consisted of 45 patients with typical effort angina or positive treadmill exercise testing but no episodes of angina at rest. All patients with angina had >75% stenotic lesions in the left coronary artery determined by myocardial perfusion scintigraphy to be the culprit lesion. The non-CAD group consisted of 24 patients with chest pain syndrome (n=22) or mitral valve prolapse (n=2). They had no significant coronary artery stenosis >25% luminal diameter. No patients had acute infection, acute inflammation, or psychological disorders. No patients had taken antidepressant drugs, major tranquilizers, steroids, or nonsteroidal antiinflammatory drugs except for aspirin. Written informed consent was obtained from all patients before enrollment in the study.

Human Blood Samples

Before the injection of a contrast medium, blood samples were collected from the coronary sinus (Cs), aortic root (Ao), and femoral vein. At the time of blood sampling, the first 3 mL of blood was discarded, and additional blood was drawn into a tube containing EDTA (pH 7.5) for NT assay. The blood samples were immediately centrifuged at 3000 rpm for 10 minutes at 4°C, and the plasma was stored at −80°C until assayed.

Measurement of Plasma NT Levels

NTs were measured by sandwich ELISAs according to the manufacturer's instructions for BDNF and NT-3 (Promega). Assays were performed on polystyrene 96-well plates. The NT concentration was quantified against a standard curve calibrated with known amounts of protein. The detection limits were 4 pg/mL for BDNF and 8 pg/mL for NT-3. The BDNF or NT-3 ELISA systems have very low cross-reactivity with other related neurotrophic factors: 3% or 0.11% cross-reactivity, respectively. Each value is a mean of duplicated measurement.

Human Tissue and Immunohistochemistry

Human coronary arteries were collected from 11 autopsy cases within 6 hours after death. Table 2 shows the characteristics of the autopsy cases. Coronary arteries were removed from the heart and cut into 3-mm lengths. Before the immunohistochemical analysis, all autopsy sections were examined by hematoxylin and eosin staining and classified into nonatherosclerotic coronary arteries (n=7) and atherosclerotic arteries (n=13). Coronary specimens were obtained from patients with SAP (n=29) or UAP (n=21) during DCA.

Tissue distribution of BDNF was detected through the use of immunohistochemical methods according to the manufacturer's instructions for anti-human BDNF antibody (Chemicon International or Santa Cruz Biotechnology Inc). BDNF antibody has <0.1% cross-reactivity with recombinant human NT-3 or NT-4/5. Human tissues were fixed in Zamboni's fixative (4% formaldehyde, 15% picric acid in 0.1 mol/L phosphate buffer) for 2 hours and in 30% sucrose in PBS overnight at 4°C. Cryostat sections (20 μm) were blocked with 20% normal horse serum in PBS for 1 hour, followed by incubation with primary antibody diluted 1:500 in 2× PBS and

TABLE 2. Characteristics of Autopsy Cases

Sex	Age, y	Cause of Death	Associated Cardiovascular Disease(s)
Male	82	Hepatoma	Stable angina
Male	78	Pancreatic cancer	Hypertension
Male	73	Cardiac rupture	Acute myocardial infarction
Male	70	Congestive heart failure	Old myocardial infarction
Female	66	Hepatoma	Hypertension
Male	65	Lung cancer	None
Female	64	Lymphoma	Hypertension
Male	63	Colon cancer	None
Male	51	Congestive heart failure	Dilated cardiomyopathy
Male	41	Sudden death	Ventricular tachycardia
Male	35	Gastric cancer	None

0.3% Triton X-100 containing 0.02% sodium azide. After a 24-hour incubation at room temperature, the samples were washed and incubated with biotinylated goat anti-rabbit immunoglobulin (DAKO). For color development, we used an LSAB kit (DAKO). For a negative control, the primary antibody was replaced with rabbit serum.

Double-Labeling Immunofluorescence

The antibodies used in double staining were mouse monoclonal anti-human CD68 antibody (DAKO) for macrophages and mouse monoclonal anti-human smooth muscle α-actin antibody (DAKO) for smooth muscle cells. Texas red–conjugated anti-mouse immunoglobulin and FITC-conjugated anti-rabbit immunoglobulin were applied as secondary antibodies. The samples were examined by a laser scanning confocal imaging system (MRC-1024, Bio-Rad Laboratories).

Semiquantitative Analysis of BDNF in Immunohistochemistry

According to a previous study, the expression of BDNF in each DCA specimens was graded as follows: grade 0=negative stain, grade 1=variable or weak stain, and grade 2=moderately or strongly positive stain.[18] The sections were blindly graded by 3 independent senior pathologists.

Measurement of NAD(P)H Oxidase Activity in Human Coronary Artery Smooth Muscle Cells

Human coronary artery smooth muscle cells (CASMCs; Clonetics) were cultured with medium (Clonetics) supplemented with 10% FBS and the manufacturer's reagents (Clonetics).

The enzymatic activity of NAD(P)H oxidase in human CASMC homogenates was assessed by lucigenin-enhanced chemiluminescence (L-CL). Human CASMCs were preincubated with or without 100 ng/mL BDNF for 1 hour; suspended in homogenate buffer containing 50 mmol/L Tris/HCL (pH 7.4), 1.0 mmol/L EDTA, 500 mmol/L phenylmethylsulfonyl fluoride, 2.0 mmol/L leupeptin, and 2.0 mmol/L pepstatin A; and then homogenized with an ultrasonicator (4×15 seconds) on ice. In the time-course study, the cells were incubated for each period with 100 ng/mL BDNF; in the dose-response study, they were stimulated with the indicated concentration of BDNF for 1 hour. The assay solution contained 50 mmol/L HEPES (pH 7.4), 1.0 mmol/L EDTA, 6.5 mmol/L $MgCl_2$, 5.0 μmol/L lucigenin as an electron acceptor, and either 1 mmol/L NADH or 1 mmol/L NADPH as a substrate. After preincubation at 37°C for 10 minutes, the reaction was started by adding 100 μL cell homogenate. The final volume of the reaction solution was 1.0 mL. Photon emission was recorded continuously for 20 minutes. The chemiluminescent signals observed in the absence of homogenates were subtracted from the chemiluminescence signals of the samples. The chemiluminescence signal was corrected for the protein concentration of each cell homogenate and expressed as counts per minute per milligram protein for an average 20-minute period. In some experiments, the cell homogenates were preincubated with 10 μmol/L diphenylene iodonium (DPI), a selective NAD(P)H oxidase inhibitor, for 20 minutes before L-CL measurement.

Superoxide Production From Human CASMCs

Dihydroethidium oxidative fluorescence dye was used to evaluate in situ production of superoxide. Human CASMCs were preincubated with or without 100 ng/mL BDNF for 1 hour, treated with dihydroethidium (10 μmol/L), and then capped with coverslips. The slides were incubated in a light-protected humidified chamber at 37°C for 20 minutes. The dihydroethidium image was obtained by a laser scanning confocal imaging system (MRC-1024) equipped with a 585-nm long-pass filter.

TABLE 3. BDNF and NT-3 Concentration in Cs, Ao, and Peripheral Vein

	Non-CAD, pg/mL	SAP, pg/mL	UAP, pg/mL	*P*
BDNF				
Cs	1308 (822–1711)	1206 (683–1608)	1503 (1123–2350)	0.67
Ao	1347 (781–1743)	1229 (697–1705)	1249 (776–1754)	0.53
V	1297 (654–1856)	1267 (781–1706)	1304 (802–1689)	0.78
NT-3				
Cs	511 (460–555)	459 (380–546)	433 (308–807)	0.51
Ao	498 (345–587)	428 (360–530)	430 (332–753)	0.76
V	492 (387–606)	445 (365–530)	442 (321–799)	0.77

V indicates peripheral vein. All values are given as medians and interquartile ranges. Probability values are for the comparison among the 3 groups.

Statistical Analysis

Data are expressed as medians and interquartile ranges, medians and ranges, or means and SEM as appropriate. Statistical comparison for categorical variables such as risk factors and sex was performed by the χ^2 test. Age and left ventricle ejection fraction were compared between groups with 1-way ANOVA. Statistical comparison for coronary stenosis between the SAP and UAP groups and the NAD(P)H oxidase activity was performed by the nonparametric tests; the Mann-Whitney was used for comparison. Statistical comparison for NT plasma levels was performed by the nonparametric Kruskal-Wallis tests with multiple-comparison post-hoc procedures (Dunn's method). Statistical analysis was performed with StatView 5 software (SAS Instituite, Inc). A value of $P<0.05$ was considered to indicate statistical significance.

Results

Patient Characteristics

Table 1 shows the clinical characteristics of the 3 groups. There were no statistical differences among the 3 groups in the following variables: age, sex, left ventricular ejection fraction, hypertension, diabetes mellitus, hyperlipidemia, smoking, or obesity. There were no significant differences between the UAP and SAP groups in the number of diseased vessels or the degree of angiographic stenosis. There was no difference in the standard medications between the UAP and SAP patients.

BDNF in Coronary Circulation Was Increased in Unstable Angina

There was no difference in plasma levels of BDNF and NT-3 among the 3 groups (Table 3). To examine coronary circulation–specific levels of NTs, the difference in NT levels between the Cs and Ao was calculated. The Cs-Ao difference in plasma BDNF in the UAP group was significantly greater than that in the SAP and non-CAD groups, whereas there was no Cs-Ao difference in NT-3 among the groups (Figure 1).

TABLE 4. Semiquantitative Analysis of BDNF in Coronary Specimens Obtained by DCA

	Expression Scores
SAP (n=29)	0.57 (0.33–1.33)
UAP (n=21)	1.74 (1.0–2.33)*

For expression scores, negative stain=0, weak stain=1, and moderate or strong stain=2. All values are given as medians and interquartile range.
*Significant differences between UAP and SAP, $P<0.01$.

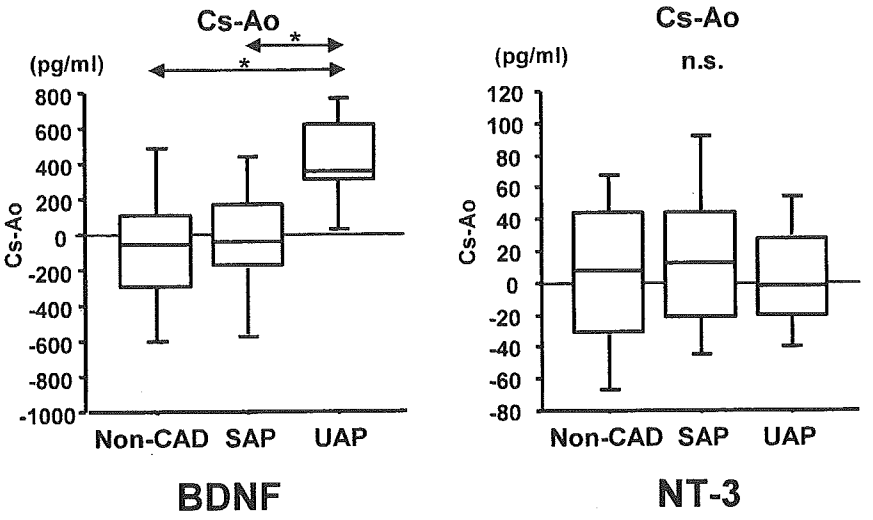

Figure 1. Cs-Ao differences in plasma NT levels across the coronary circulation. The Cs-Ao differences in plasma BDNF were significantly greater in the UAP group than in the SAP or non-CAD group, whereas the Cs-Ao differences in NT-3 were not significantly different among the 3 groups. Data are expressed as medians, with 25th and 75th percentiles (boxes) and 10th and 90th percentiles (I bars). *$P<0.01$ for the comparisons of the UAP, SAP, and non-CAD groups.

These findings indicate that the generation of BDNF in the coronary circulation was enhanced in patients with UAP.

BDNF Expression in Human Coronary Artery

The blood sampling results (Figure 1) led us to examine BDNF expression in the coronary arteries. Immunohistochemical analysis of coronary arteries obtained from autopsy cases was performed. BDNF was expressed in atherosclerotic coronary arteries in all specimens (Figure 2). BDNF was preferentially localized in the atheromatous intima and around the vasa vasorum in the adventitia (Figure 2D and 2E). In contrast, BDNF immunoreactivity was barely detected in nonatherosclerotic coronary arteries (Figure 2C).

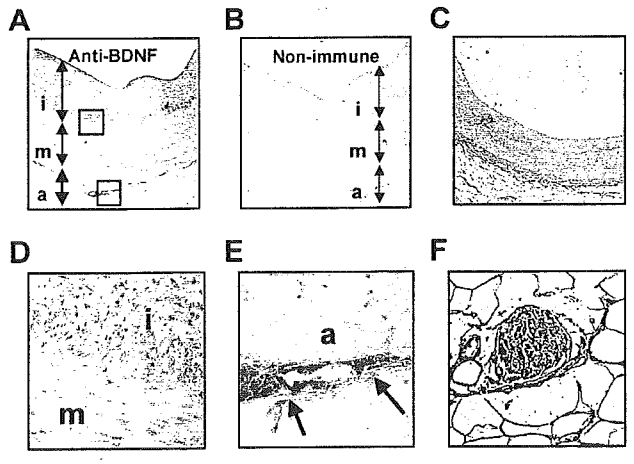

Figure 2. BDNF expression in human coronary arteries of autopsy cases. A, Low-power view of representative human atherosclerotic coronary arteries obtained from autopsied cases shows intense BDNF immunoreactivity in the atherosclerotic intima (i) and adventitia (a). B, There is no significant staining with nonimmune serum used as a control. C, Low-power view of representative nonatherosclerotic coronary arteries shows negligible immunoreactivity of BDNF. D, High-power view of the area indicated by the rectangle in A shows BDNF expression in smooth muscle cells of the intima. E, High-power view of the area indicated by the rectangle in B shows the expression of BDNF in fibroblasts around the vasa vasorum in the adventitia. F, Immunostaining of BDNF in peripheral nerves in pericardial tissues. m Indicates media.

Figure 2F shows BDNF expression in peripheral nerves. Double staining with cell-specific markers using serial sections revealed that some smooth muscle cells and macrophages expressed BDNF (Figure 3).

BDNF Expression in Coronary Specimens of Patients With Angina Pectoris

Investigation of coronary specimens from DCA of patients with SAP (n=29) and UAP (n=21) revealed enhanced BDNF expression in inflammatory cells, smooth muscle cells, and extracellular matrix (Figure 4). To investigate the

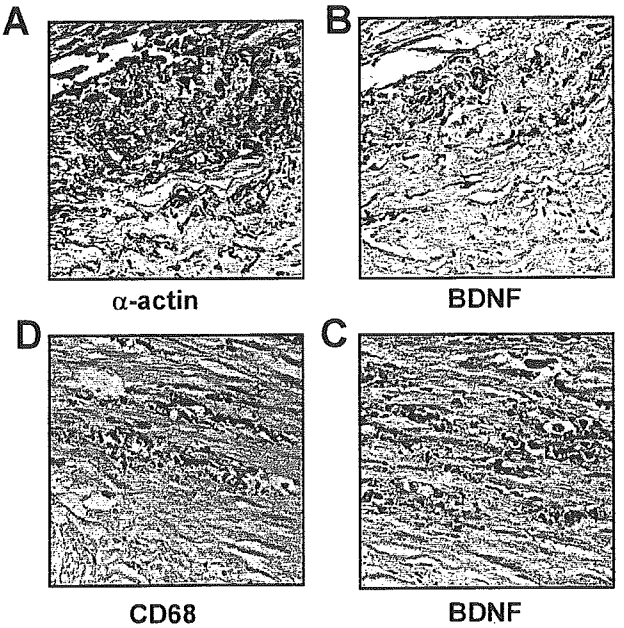

Figure 3. Association between BDNF and CD-68 or α-actin in atherosclerotic coronary arteries. A, B, Immunohistochemical staining of α-actin, a marker of smooth muscle cells (A), and BDNF (B) in serial sections of atherosclerotic coronary arteries of autopsy sample, showing that some smooth muscle cells expressed BDNF. C, D, Immunohistochemical staining of CD-68, a marker of macrophages (C), and BDNF (D) in serial sections of atherosclerotic coronary arteries of autopsy samples, showing that macrophages expressed BDNF.