1．アテローム血栓症の機序

2) 内皮機能と血栓症

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THROMBOSIS and Circulation

§論文のポイント

[1] 血管内皮細胞はNOをはじめとした種々の分子を産生分泌し，血液凝固や血小板凝集の調節に働いている．

[2] 血液の流動性を保ち，血栓が生じないように機能している血管内皮が傷害されること，あるいはもっと進行した形として内皮の剥離を生じることが，アテローム血栓症の発症には必須である．

[3] 生体において，内皮機能障害を判定するには大きく分けて2通りの方法がある．1つはVWFのような，内皮が傷害を受けた際に血液中に放出される物質(マーカー)を測定する手法であり，もう1つは，代表的な内皮機能である内皮依存性の血管弛緩・拡張反応（EDR)を測定するものである．

[4] EDRにて判定した血管内皮機能障害の程度が，冠動脈疾患患者の心血管イベント発症に関係するとの報告がいくつかなされており，内皮機能障害がアテローム血栓発症の基盤になることを示すものと理解される．

§キーワード

血管内皮／血栓形成／一酸化窒素／von Willebrand因子／内皮依存性血管弛緩・拡張反応

はじめに

血管内皮は多彩な機能を有しており，血管のホメオスタシスの維持に必須な「臓器」であり，また，血管の炎症，リモデリングの場として中心的な役割を果たしている．そして内皮細胞は，一酸化窒素(NO)をはじめとした種々の分子を産生分泌し，血液凝固や血小板凝集の調節に働いている(表1)[1]．

生体においては，抗血栓・凝固因子と向血栓・凝固因子とがバランスのとれた状態で作用しあい，全体として血液の流動性を保っている．この，抗血栓・凝固作用の場の中心となっているのが血管内皮であり，内皮が傷害あるいはそのさらに進んだ形として剥離されることにより，抗血栓・凝固から，向血栓・凝固へとスイッチが生じ，血栓が形成されるものと考えられる．

表1　内皮細胞が産生する凝固・血栓形成に関係する因子

1．抗凝固・血栓形成因子

○抗凝固因子
- アンチトロンビン
- 外因系経路阻害因子(tissue factor pathway inhibitor ： TFPI)
- トロンボモデュリン

○抗血小板粘着・凝集因子
- 一酸化窒素(NO)
- プロスタサイクリン(PGI_2)
- ecto-ATPDase

○線溶系因子
- 組織型プラスミノゲン・アクチベータ(t-PA)

2．向凝固・血栓形成因子

○向凝固因子
- 組織因子(tissue factor ： TF)

○向血小板粘着・凝集因子
- 血小板活性化因子(platelet activating factor ： PAF)
- von Willebrand 因子(VWF)

○線溶阻害因子
- プラスミノゲン・アクチベータ・インヒビター-1 (PAI-1)

内皮細胞から産生される抗血栓・凝固因子

内皮細胞が産生する抗凝固因子の代表としては，抗血栓形成に関してはNOとプロスタサイクリン(PGI_2)が挙げられる．NOは内皮細胞の内皮型NO合成酵素(eNOS)が血流のずり応力により活性化されることにより，常に産生されている．またeNOSは，アセチルコリンやブラジキニンなどのG蛋白質共役型アゴニストの刺激による細胞内カルシウム増加により活性化されNOを産生する．さらに，血中のインスリンやVEGFによってeNOSがリン酸化されることもNO産生には重要である．NOは細胞内の可溶性グアニル酸シクラーゼを活性化し，サイクリックGMP (cGMP)を増加させる．血管においては，平滑筋細胞内cGMPを増加させることにより血管平滑筋を弛緩・拡張させる(内皮依存性血管弛緩・拡張反応)．それとともに，血小板にも作用し，血小板の凝集と粘着を抑制する[2]．一方，PGI_2は，アラキドン酸よりプロスタサイクリン合成酵素によって産生され，血小板の受容体を介し，細胞内サイクリックAMP (cAMP)濃度を上昇させることにより，血小板の凝集と粘着を抑制する．

内皮細胞が産生する抗凝固因子としては，まずトロンボモデュリン(TM)が挙げられる．TMは，トロンビンに結合することでその立体構造を変え，トロンビンがフィブリノゲンと反応することを阻害する．さらにトロンビン・トロンボモデュリン複合体はプロテインCの生理活性を数万倍にも増強し，抗凝固に働く[3]．一方，アンチトロンビンは内皮細胞表面のヘパリン用物質と相互作用し，トロンビンなどの凝固因子の作用を阻害するとともに，白血球の活性化も抑制している．さらには，内皮細胞からのプロスタサイクリン産生を促進する．外因系経路阻害因子(tissue factor pathway inhibitor ： TFPI)は，プロテアーゼインヒビターであり，組織因子(TF)による血液凝固の外因性反応を阻害し，また，内皮や平滑筋細胞の増殖を抑制する作用を有する．さらに内皮細胞は，組織プラスミノゲンアクチベータ(t-PA)を産生し線溶を活性化している[4]．

内皮細胞が産生する向血栓・凝固因子

内皮が産生する代表的血栓形成因子としてはvon Willebrand因子(VWF)と，TFが挙げられる．VWF

は内皮細胞と骨髄巨核球において選択的に産生されており，巨核球で産生されたVWFは血小板のα顆粒に蓄積され，刺激により血中に分泌される．内皮で産生されたVWFはそのまま放出される一方で，分泌顆粒であるWeibel-Palade体に貯蔵され，刺激に応じて放出される．血中に存在するVWFの大部分は内皮由来である．大動脈などにおけるゆるやかな血液の流れの環境下では，VWFは担体蛋白として血液凝固第Ⅷ因子を安定化させている．一方において，VWFは動脈硬化粥腫による狭窄部位のような高いずり応力，すなわち血流の速い部位において，血小板を細胞外マトリックスに付着させるために重要な役割を果たしている[5]．VWFはコラーゲンの表面において固相化され，血小板とGPIb/Ⅸ/V複合体を介して結合する．この反応により血小板は血管傷害の部位に集積する．この結合は一過性であり，また高いずり応力の存在下でのみ生じる現象である．こうしてコラーゲン表面に結合した血小板は，コラーゲン受容体，ADP受容体などから刺激を受け活性化し，活性化GⅡb/Ⅲaを介してフィブリノゲン，VWFと長時間結合が可能となり，血小板血栓の3次元的な成長が生じる．

一方，TFは血液と接しない血管細胞，平滑筋細胞や線維芽細胞の表面には常に発現しており，血管が傷害されると血液中のⅧ因子と複合体を形成し，血液凝固の外因性反応を惹起する．TFは定常状態の内皮細胞には発現していないが，炎症などの種々の刺激により内皮細胞が活性化されることにより，内皮にもTFが発現する[6]．

内皮傷害と血栓形成

血液の流動性を保ち，血栓が生じないように機能している血管内皮が傷害されること，あるいはもっと進行した形として内皮の剥離を生じることが，アテローム血栓症の発症には必須である．すなわち，動脈硬化粥腫が破綻すると内皮の剥離が生じ，内皮下の血栓性マトリックスが血液に曝露される．その結果血小板は活性化され，VWFを介し損傷部位のマトリックスに結合・集積し，血小板血栓の形成が始まる．また，血小板血栓形成部位の下流では，TFによって凝固系が活性化し，フィブリンの形成とともに血栓が発育していく．

一方，内皮細胞の剥離が生じなくても，高脂血症や，糖尿病，高血圧，喫煙などの病態下では，酸化ストレス，炎症，酸化LDLなどにより，血管内皮は傷害されている．傷害を受けた内皮では，内皮細胞からのNOやプロスタサイクリンの産生が減弱する．さらには傷害を受けた内皮は活性化され，VWF産生の増加，内皮でのTFの発現，血小板の活性化をもたらし，血栓形成が生じやすくなっている．

実際に，冠動脈危険因子に関する前向き大規模臨床試験において，血中のVWF濃度は，フィブリノゲン濃度や白血球数とならんで，冠動脈疾患発症のリスクになることが示されている[7]．しかしながら，この研究では，糖尿病や高血圧，喫煙，加齢などの古典的危険因子の影響を除外すると，VWF濃度は危険因子とはなっていない．すなわち，種々の危険因子の存在する病態下では内皮傷害が生じており，その結果VWFの産生が増加し，血管が易血栓形成性へ傾いていると理解される．

それでは，生体において内皮機能障害をどのように判定すればよいのであろうか？

臨床における内皮傷害の判定

生体において，内皮傷害あるいは内皮機能障害を判定するには大きく分けて2通りの方法がある．1つはVWFのような，内皮が傷害を受けた際に血液中に放出される物質(マーカー)を測定する手法であり，もう1つは，代表的な内皮機能である内皮依存性の血管弛緩・拡張反応(EDR)を測定するものである．

1．内皮傷害を示す血中マーカー

表2に内皮細胞から産生・放出される物質の中で，内皮細胞傷害のマーカーとして使用しうるものを挙げる．すでに述べたようにVWFはその代表であり，抗原量を測定する．また，可溶性ICAM-1やVCAM-1のような内皮細胞に発現する接着因子，さらには可溶性トロンボモデュリンが有用との報告もある．可溶性トロンボモデュリンは，内皮におけるトロンボモデュリンの発現量を反映していると考えられ，米国での前向き大規模臨床試験において，その血中濃度が冠動脈疾患の発症と逆相関することが示された[8]．

また，マイクロパーティクルも注

表2　臨床における内皮傷害の判定法

1．内皮由来血中マーカーの測定
○内皮細胞由来分子
　von Willebrand 因子
　可溶性接着因子　sICAM-1，sVCAM-1
　可溶性トロンボモデュリン
○マイクロパーティクル
○血管内皮前駆細胞
2．内皮依存性血管弛緩・拡張反応の測定
　冠動脈：冠動脈造影，カテ先ドプラ血流計
　前腕動脈：エコー法，プレチスモグラフィー

目を浴びている．マイクロパーティクルは，種々の細胞が活性化されたり，あるいは傷害されることにより形成され，血中に放出される膜小胞体である．血中に存在するマイクロパーティクルは，血小板由来のものを含め多彩であるため，内皮由来のマイクロパーティクルをどのように判別するかが問題となる[9]．最近になり，VE-カドヘリンの抗体を用いて，内皮由来マイクロパーティクルを識別する方法が開発され，糖尿病患者において内皮由来マイクロパーティクルの血中濃度が上昇していること，そして冠動脈疾患の独立した危険因子となることが報告された[10]．

一方，血中に循環する内皮前駆細胞も，内皮細胞傷害のマーカーとして注目されている．内皮前駆細胞が血中に存在していることがAsaharaらにより，1997年に報告され[11]，その後，内皮前駆細胞を用いた血管新生の研究が一気に加速したのは周知の通りである．しかしながら内皮前駆細胞は，もともと傷害血管での内皮の修復に関与する細胞として発見されたのであり，内皮傷害がある場合は，その修復のため循環血液中の本細胞数が増加している可能性が考えられる．実際に糖尿病や，高血圧，喫煙などの危険因子の存在下では血中の内皮前駆細胞の数が増えていること，さらには内皮前駆細胞数が後述の前腕のEDRと相関することも報告されている[12]．

2．内皮依存型血管弛緩・拡張反応(EDR)

以上のような内皮傷害の血中マーカーがあるが，実際の臨床の場においては，内皮傷害の判定の主体はEDRの測定によってなされているのが現状であり，上述の血中マーカーが実際に内皮傷害を反映するかどうかもEDRと比較することで判定されている．対象とする血管が冠動脈の場合は，心外膜の太い冠動脈でのEDRの判定は，アセチルコリンなどの内皮依存性血管拡張物質の投与による血管径の変化を冠動脈造影により測定し，冠動脈抵抗血管でのEDRは，これらの薬剤投与時の血流変化をカテ先ドプラ血流計で測定し判定する．一方非侵襲的なEDRの検査法としては，前腕動脈における血流依存性の血管拡張反応(flow-mediated vasodilation：FMD)の測定が行われる．すなわち，マンシェットにより前腕を短時間止血し，その後再灌流する．そうすると反応性充血と称される一過性の血流量増加を生じる．この血流量の増加によりずり応力が増し，その結果eNOSが活性化され，内皮依存性の血管拡張反応が前腕動脈に生じる．この拡張反応をエコー法にて測定し，血流依存性のEDRを測定し定量化する．このEDRにて判定した血管内皮機能障害の程度が，冠動脈疾患患者の心血管イベント発症に関係するとの報告がいくつかなされており，内皮機能障害がアテローム血栓発症の基盤になることを示すものと理解される．

おわりに

血管内皮は抗血栓，抗凝固において中心的な役割を果たしている．種々の動脈硬化の危険因子やあるいは炎症は，内皮を傷害し血栓形成を誘導する．心血管イベントの多くはアテローム血栓症によりもたらされるものであるが，大規模臨床試験においてスタチン，ACE阻害薬，アンジオテンシンⅡ受容体拮抗薬などが，心血管イベントの発症を抑制することが明らかになっている．これらの薬剤はいずれも内皮機能を改善することが明らかになっており，その心血管イベント抑制作用に，内皮機能の改善による抗血栓，抗凝固作用の増強が関係していると考えられる．

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III. 発症機序

メタボリックシンドローム発症にかかわる血管内皮細胞機能障害

Endothelial dysfunction as a mechanistic factor for metabolic syndrome

川嶋成乃亮

Key words：メタボリックシンドローム，血管内皮細胞，インスリン抵抗性，アディポサイトカイン

はじめに

血管内皮細胞は数多くの分子を産生・分泌することにより，血管の緊張性の調節のみならず，血管壁の恒常性の維持，更には血液の凝固・線溶調節などの多彩な機能を有す．そして，血管内皮細胞の障害が種々の血管病の病態形成に深く関係しており，特に動脈硬化の発症・進展に必須な要因となることは広く認められている．

近年，動脈硬化発症の基盤としてメタボリックシンドロームの存在が注目を浴びており，メタボリックシンドロームにおいては，内皮機能が障害されていることが明らかになっている[1]．メタボリックシンドロームを構成する高血圧，糖尿病，脂質代謝異常のいずれもが単独においても内皮機能障害をもたらすが，メタボリックシンドロームの中心にあると考えられるインスリン抵抗性そのものもまた，内皮機能を減弱すると考えられている[2,3]．一方，メタボリックシンドロームの病態は，①内臓脂肪蓄積，②インスリン抵抗性，③脂質代謝異常，④高血圧，⑤易血栓状態，⑥易炎症状態から形成されており，内皮機能障害の存在はこれらの病態に深く関与していることが想定される．

本稿においては，メタボリックシンドロームにおける内皮機能障害の意義，およびその機序について解説するとともに，内皮細胞機能障害の存在がどのように，メタボリックシンドロームの病態形成に関与しているかについて考察する．

1. メタボリックシンドロームにおける内皮機能障害

臨床において内皮機能障害の有無は，内皮依存性の血管弛緩・拡張反応(EDR)を測定することで一般的にその判定がなされており，EDRの低下があれば，内皮機能が障害されていると考える．EDRは血流のずり応力や種々のアゴニスト刺激により，内皮細胞に存在する内皮型NO合成酵素(eNOS)が活性化され，その結果NOが産生され，拡散により血管平滑筋に達したNOがcGMP依存性に血管平滑筋を弛緩・拡張させる現象である．

今日，メタボリックシンドロームの中心をなすのは内臓肥満と考えられている．肥満は多くの場合，高血圧，糖尿病，脂質代謝異常などの他のメタボリックシンドロームの構成要因を合併しており，それら自身が内皮機能の障害要因であるため，肥満そのものの影響を判定することは困難である．一方，これらの明らかな要因を除外しても肥満に伴いEDRが減弱するとの報告も散見されるが，この場合は恐らく肥満そ

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表1 メタボリックシンドロームにおける内皮機能低下の機序

1. 酸化ストレスの増加
2. 内皮におけるインスリン抵抗性
3. アディポサイトカインの作用
   血管に対する直接作用
   内皮細胞内におけるインスリンシグナルの修飾
4. 増加した遊離脂肪酸の影響

のものというより，肥満の下流にあると考えられるインスリン抵抗性が原因と考えられる[3]．このような考え方を支持するものとして，Lteifらは，メタボリックシンドロームの患者において，下腿動脈で測定したEDRと，メタボリックシンドロームの5つの要因，すなわちウエスト周囲径，中性脂肪値，HDLコレステロール値，血圧，血糖値との関係を検討し，EDRの減弱と関係するのはウエスト周囲径と血圧であること，更にウエスト周囲径のEDRへの影響はインスリン抵抗性の指標であるHOMA-Rにより規定されているということを報告している[4]．

2. メタボリックシンドロームにおける内皮機能低下の機序

それでは，メタボリックシンドローム/インスリン抵抗性における内皮機能の低下には**表1**に示すような種々の機序の関与が考えられる[2,5]．

まずは，内皮障害を生じる種々の病態に共通の機序として，酸化ストレスの亢進があげられる．メタボリックシンドロームを構成する高血圧，糖尿病，脂質代謝異常などにおいてはスーパーオキシドの産生が亢進しており，その結果NOの不活性化が生じている．高血圧は，機械的刺激やアンジオテンシンIIなどを介し，血管におけるスーパーオキシドの主要な産生酵素であるNAD(P)H oxidaseを活性化させ，スーパーオキシドを産生する．高血糖は，ポリオール代謝系を亢進させることによるNAD(P)H oxidaseの活性化，ならびにミトコンドリア内での電子伝達系に働きスーパーオキシド産生をもたらす．また高脂血症，高血糖においては，eNOS機能に必須な補酵素であるテトラヒドロビオプテリン(BH_4)が酸化などにより組織濃度が低下する一方，BH_4の作用に拮抗するBH_2が増加することにより，eNOSの機能異常(アンカップリング)が生じ，その結果eNOSがスーパーオキシドを産生するようになっている[5,6]．酸化ストレスがメタボリックシンドロームにおける内皮機能障害に関与していることは，抗酸化薬であるビタミンCにより内皮機能が改善することからも示される[7]．

一方，メタボリックシンドロームに特徴的な機序として，まずインスリンそのものの，内皮細胞のeNOS/NO系に対する作用の減弱があげられる．インスリンによる血管拡張には，交感神経を介する間接的な作用と，eNOSに作用しNOを産生することによる内皮依存性の直接作用とがある．インスリンは内皮のインスリン受容体と結合した後，PI3 kinase/Akt経路を介しeNOSのSer1177をリン酸化しeNOSを活性化させる．また，インスリンはeNOS mRNAを増加させることも明らかとなっている[8]．そして，インスリン抵抗性を伴う肥満患者は，痩身者に比べ，血管に内皮細胞のインスリンに対する血管拡張反応が減弱していることが示されている[9]．NOが内皮保護因子として作用していることを考えると，このインスリンによるNO産生の減弱は，単に血管拡張性の減弱のみならず，広く内皮機能障害と結びつくものと考えられる．メタボリックシンドロームにおけるこのインスリンの内皮におけるシグナル伝達の障害の機序としては，アディポサイトカインの分泌異常が関係していることが示唆されている．

また，インスリン抵抗性の上流に存在する内臓肥満をもたらす脂肪細胞により産生される様々な生理活性物質，すなわちアディポサイトカインの分泌異常もまた，内皮機能障害に関与しているものと考えられる．代表的なアディポサイトカインであるアディポネクチンの血中濃度はメタボリックシンドロームで低下している．アディポネクチンには抗炎症作用，抗動脈硬化作用があるが，その機序の一つとして内皮保護作用が重要である．アディポネクチンはEDRをもたらすが，これはAMPK(AMP-activated protein kinase)を介した経路およびAktを介し

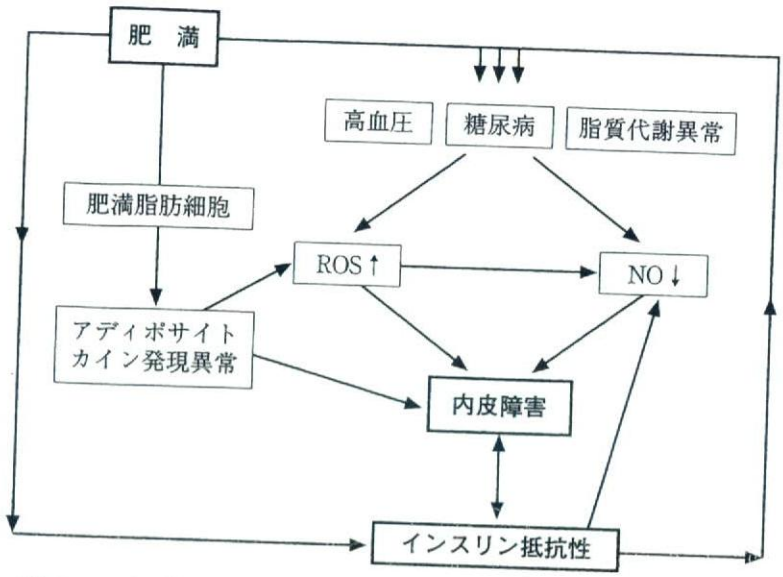

図1　メタボリックシンドロームにおける内皮障害の成立機構

た経路により，eNOSをリン酸化し活性化させNOを産生することによる[10]．また後ほど述べるように，アディポネクチンは内皮細胞における接着因子発現を抑制している．一方，メタボリックシンドロームで増加しているTNFαやレジスチンは内皮の障害因子として働く[11]．TNFαにはeNOS発現の抑制作用があり，TNFα，レジスチンには血管収縮因子であるET(エンドセリン)-1の発現の増強作用がある．このように，内皮保護因子であるアディポネクチンが低下し，障害因子であるTNFαやレジスチンが増加していることが，メタボリックシンドロームにおける内皮機能障害に働いていると考えられる(**図1**)．

3. 内皮障害がメタボリックシンドロームの病態形成に及ぼす役割

近年，動脈硬化を慢性炎症性疾患ととらえる考え方が広まりつつある．その意味において，メタボリックシンドロームにおいて認められる易炎症性，そして易血栓性はメタボリックシンドロームが動脈硬化の発症基盤となるうえで重要である．メタボリックシンドロームでは前述のようにアンジオテンシンIIやエンドセリン，更に高血圧といった機械的因子の影響でスーパーオキシドの産生が増加し酸化ストレスが増大している．酸化ストレスは内皮を障害・活性化し，炎症を惹起する．その作用機序として特に重要なのは転写因子NFκBを活性化することであり，その結果VCAM-1，ICAM-1のような接着因子，更にはマクロファージ走化因子であるMCP-1，そしてmatrix metalloproteinases (MMPs)などの動脈硬化発症・進展の必須遺伝子を誘導する．更にこれらに加え，メタボリックシンドロームにおいて脂肪細胞から産生されるTNFαもまた，VCAM-1などの接着分子の発現，あるいはMCP-1の発現を増加させ，炎症惹起性に作用する[12]．これに対し，内皮細胞より産生されるNOはスーパーオキシドの作用と拮抗し，炎症抑制に作用する．この主たる機序はNFκBの活性化の抑制によるものであり，NOにより接着因子発現が抑制され，そして内皮細胞-単球・炎症細胞接着の抑制が生じる．更にMCP-1やMCSFといった動脈硬化惹起性分子の発現も抑制する[13]．

一方，メタボリックシンドロームのもう一つの病態である易血栓性に関してであるが，この場合も酸化ストレスの増大が関係する．酸化ストレスにより内皮細胞ではNFκBの活性化を通じてプラスミノゲン・アクチベータインヒビター-1(plasminogen activator inhibitor-1: PAI-1)の発現が誘導され，一方において血液凝固の外因性のイニシエーターである組織因子(tissue factor: TF)の発現が増加する．更に血中のPAI

-1濃度が上昇しており，これは内臓脂肪細胞からの産生・分泌が主として関与する[14]．他方，内皮細胞からは抗血小板凝集・粘着因子でもあるNOやプロスタサイクリンをはじめ，アンチトロンビン，組織型プラスミノゲン・アクチベータ(t-PA)，更には外因経路阻害因子(tissue factor pathway inhibitor: TFPI)などの様々な抗凝固・血栓形成因子が産生されている．

メタボリックシンドロームにおいては，易炎症因子，易血栓形成因子が増加するとともに，内皮細胞が障害されていることにより，NOをはじめとした抗炎症因子，抗血栓形成因子が低下しているため，血管壁での炎症機転が惹起され，更に易血栓性となっていると考えられる．

このように内皮細胞機能障害の存在は，メタボリックシンドロームの病態形成に深く関与しているが，一方において内皮機能の障害が，メタボリックシンドローム発症そのものにかかわっている可能性も考えられる．すなわちメタボリックシンドロームの病態の中心に位置するインスリン抵抗性を内皮細胞機能障害が増悪させることが考えられる[15]．ACE阻害薬，およびアンジオテンシン受容体拮抗薬は，高血圧の治療薬として用いられるが，大規模臨床試験を通じてこれらの薬剤がII型糖尿病の発症を防止することが明らかになった[16]．そしてこれらの薬剤が低下したEDRを改善することより，インスリン抵抗性の出現から糖尿病発症に至る過程における，NO/内皮機能の役割が注目されるようになった．インスリンによるグルコース取り込みの40％が血流依存性であり，NO/内皮機能の低下は，インスリンによるグルコース取り込みの障害をもたらすと考えられる[17]．また，動物実験にて，NO合成酵素阻害薬であるL-NMMAの長期投与によりインスリン抵抗性が出現することや，内皮型NO合成酵素の遺伝子欠損マウスでも，インスリン抵抗性が存在することが報告されている[18]．

このように，インスリン抵抗性を基盤とするメタボリックシンドロームは内皮機能を障害し，一方，内皮機能障害は更にインスリン抵抗性を増強させるという一種の悪循環が存在すると想定される．

4. メタボリックシンドロームにおける内皮機能障害の改善

冠動脈の内皮機能障害の程度が高度の患者では，軽度の患者に比べ，その後の心血管イベントの発症が多いというプロスペクティブ研究からも明らかなように，内皮機能の障害は，血管病，特に動脈硬化の発症・進展に深く関与する．そして当然なことであるが，メタボリックシンドロームにおける，動脈硬化の進展，心血管イベントの発症においても，内皮機能の低下・障害が重要な役割を果たす．それゆえに内皮機能障害の改善を目指した治療戦略が重要となってくる．

メタボリックシンドロームの発症は生活習慣と深く関係しているため，生活習慣を改善することが重要であるが，生活習慣への介入により，高血圧，糖尿病，脂質代謝異常が改善することが内皮機能の改善に結びつくことはもとより，肥満の改善もまた独自に内皮機能の改善に関与する可能性も指摘されている．

ACE阻害薬やアンジオテンシン受容体拮抗薬は，高血圧，心不全，高脂血症などの種々の病態における減弱したEDRを改善し，内皮保護に働く．これらの薬剤は，メタボリックシンドロームにおいても同様に，内皮保護に働くことが明らかになっている[19]．更に，これらの薬剤はインスリン抵抗性を改善することも報告されている．このことは，メタボリックシンドロームにおける内皮機能障害，そしてインスリン抵抗性そのものにも，アンジオテンシンIIおよびアンジオテンシンIIにより産生されるスーパーオキシドが関与していることを示唆する．一方，インスリン抵抗性を改善する薬剤が，メタボリックシンドロームにおいて，内皮保護に働くことが判明しつつある．なかでもPPARγアゴニストであるチアゾリジン誘導体は，メタボリックシンドロームにおいて血中のアディポネクチン濃度を増加させ，一方TNFα濃度は低下させる．更に，内皮細胞にはPPARγが発現しており，その刺激によりeNOS発現の増加，

eNOSの活性化が生じることも明らかになっている．チアゾリジン誘導体は糖代謝改善作用に加えてこのような多彩な作用により，内皮機能を改善する．またメトホルミンも，インスリン抵抗性を改善するとともに前腕の抵抗血管における内皮機能の改善を伴うことが報告されている．

おわりに

このように，メタボリックシンドロームにおいて亢進した酸化ストレスや，あるいは内皮細胞自身のインスリン抵抗性によって内皮障害がもたらされるが，内皮障害の存在がまた，メタボリックシンドロームの病態を悪化させ，その最大合併症である動脈硬化病変の進展へと結びつける．メタボリックシンドロームの治療においては，内皮障害防止の観点に立った治療戦略が重要と考えられる．

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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 Institiute, 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, $P<0.01$.

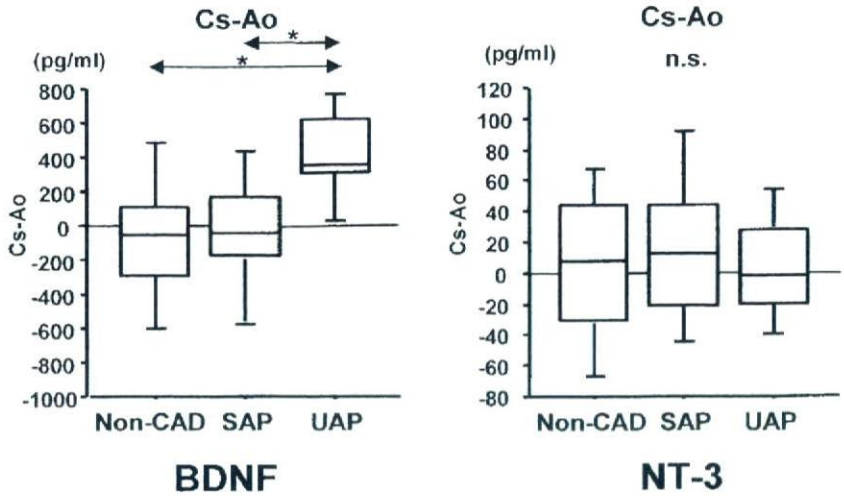

Figure 1. Cs-Ao differences in plasma NT levels across the coronary circulation. The Cs-Ao differences in plasma BDNF were significantly greater in the UAP group than in the SAP or non-CAD group, whereas the Cs-Ao differences in NT-3 were not significantly different among the 3 groups. Data are expressed 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).

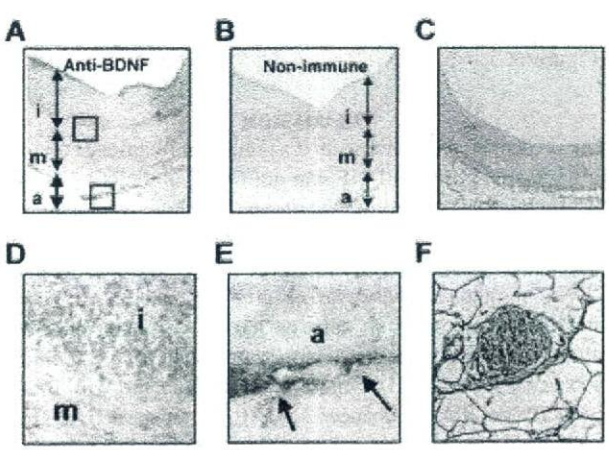

Figure 2. BDNF expression in human coronary arteries of autopsy cases. A, Low-power view of representative human atherosclerotic coronary arteries obtained from autopsied cases shows intense BDNF immunoreactivity in the atherosclerotic intima (i) and adventitia (a). B, There is no significant staining 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

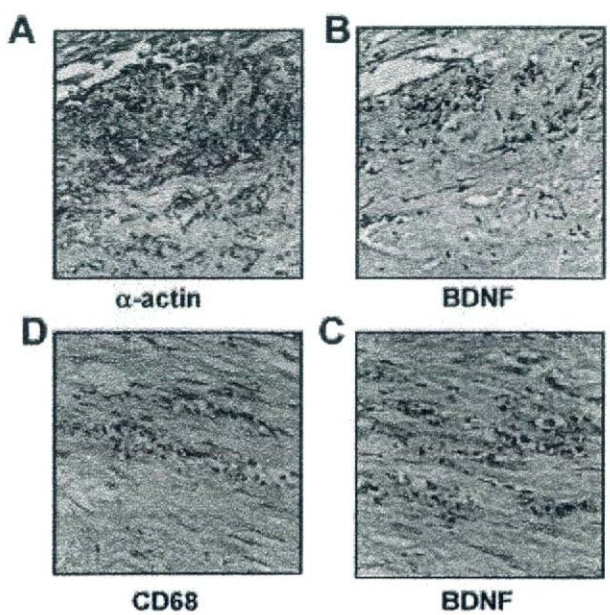

Figure 3. Association between BDNF and CD-68 or α-actin in atherosclerotic coronary arteries. A, B, Immunohistochemical staining of α-actin, a marker of smooth muscle cells (A), and BDNF (B) in serial sections of atherosclerotic coronary arteries of autopsy sample, showing that some smooth muscle cells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

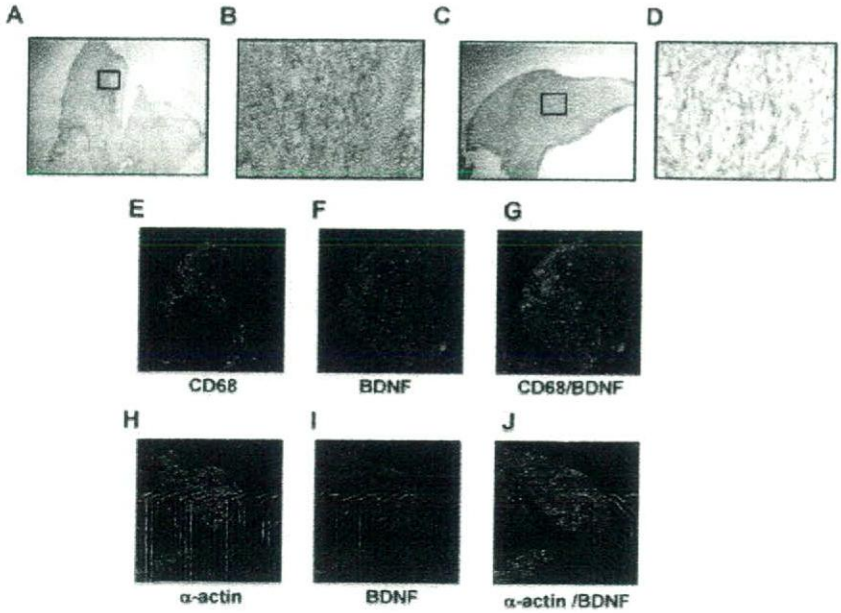

Figure 4. BDNF expression in DCA specimens. Low-power (A, C) and high-power (B, D) views of representative staining for BDNF of DCA specimens from angina patients. Immunohistochemical analysis revealed BDNF expression in inflammatory cells, smooth muscle cells, and extracellular matrix. E–J, Double immunofluorescence of BDNF with cell-specific markers. Anti-CD68 (E) and anti–α-actin (H) were used as markers of macrophage and smooth muscle cells, respectively. Red-labeled immunofluorescence indicates cell makers; green-labeled immunofluorescence indicates expression of BDNF (F, I). Colocalization of cell-specific makers and BDNF is shown by yellow-labeled immunofluorescence (G, J). Representative figure (n=5) is shown, and similar results were observed in all examinations.

cell types of BDNF-expressing cells, double immunofluorescence of BDNF and CD68 or α-actin, a specific marker of macrophages and smooth muscle cells, respectively, was carried out. As shown in Figure 4E through 4J, smooth muscle cells and macrophages expressed BDNF in coronary specimens of patients with angina pectoris.

Semiquantitative analysis demonstrated that BDNF expression in coronary arteries of UAP patients was more intense compared with SAP (Table 4). Representative cases of UAP and SAP are shown in Figure 5.

BDNF Enhanced NAD(P)H Oxidase Activity and Reactive Oxygen Species in Human CASMCs

The effects of BDNF on NAD(P)H oxidase activity were examined in human CASMCs. Stimulation with 100 ng/mL BDNF increased NADH- and NADPH-dependent oxidase activity ≈2.7- and 2.3-fold compared with that in control (nontreated) cells, respectively (Figure 6A). This oxidative activity was reduced by 10 μmol/L DPI, a selective inhibitor of NAD(P)H oxidase. In the presence of DPI, NADH- and NADPH-dependent oxidase activity in CASMCs was reduced by 74% and 45%, respectively. The effects of BDNF on NAD(P)H oxidase activity were dose and time dependent (Figure 6B). Furthermore, in experiments with dihydroethidium, an intracellular fluorescence probe, BDNF stimulation increased the generation of reactive oxygen species (Figure 6C).

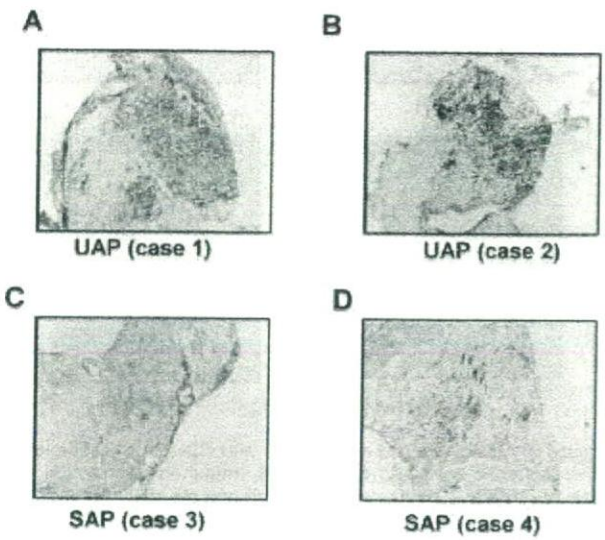

Figure 5. BDNF expression in coronary specimens of patients with angina pectoris. A B, Representative cases of UAP; C, D, representative cases of SAP. Semiquantitative analysis is shown in Table 4.

Discussion

The present study demonstrated a significantly greater Cs-Ao difference in plasma BDNF, but not NT-3, in the UAP group than in the SAP and non-CAD groups. Immunohistochemical analysis revealed that BDNF was expressed in atheromatous intima and adventitia in human coronary arteries. Intense BDNF immunoreactivity was observed in macrophages and smooth muscle cells in atherosclerotic coronary arteries. Semiquantitative analysis demonstrated that BDNF expression in UAP patients was more intense compared with SAP. Furthermore, BDNF enhanced NAD(P)H oxidase activity and superoxide production in cultured CASMCs, and its selective inhibitor suppressed the effect of BDNF. Thus, BDNF in the coronary vasculature might enhance oxidative stress via the activation of NAD(P)H oxidase.

BDNF has protective effects against injury or ischemia in both the central and peripheral nervous systems.[4–6] For example, Schabitz et al[19] demonstrated that intravenous BDNF injection reduces infarct size in rat model. On the other hand, several lines of evidence suggest that NTs potentiate neuronal death under some conditions such as serum or oxygen-glucose deprivation.[20,21] Kim et al[17] demonstrated that BDNF acts as a pronecrotic factor through activation of NAD(P)H oxidase in cortical cells, and other

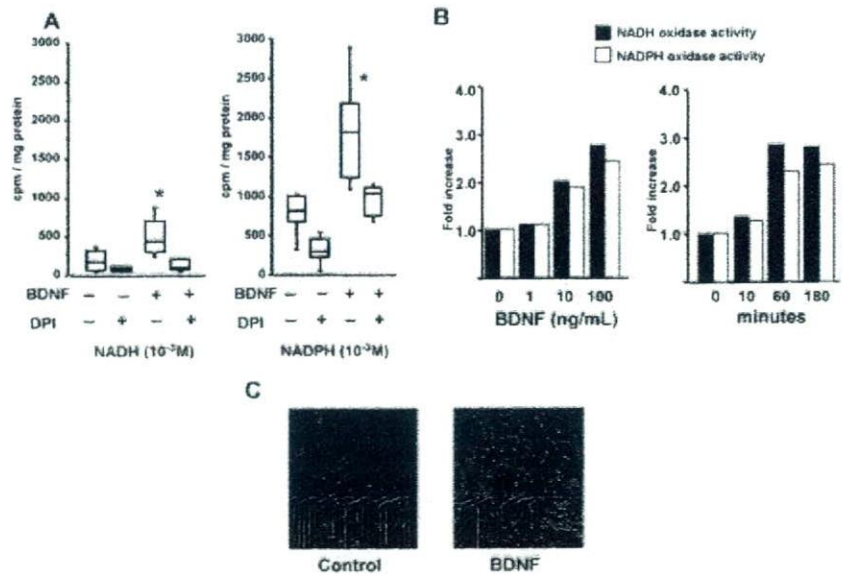

Figure 6. A, Effect of BDNF on NAD(P)H oxidase in cultured human CASMCs. Enzymatic activity of NAD(P)H oxidase in human CASMC homogenates was assessed by L-CL. NADH- (left) and NADPH- (right) dependent oxidase activity was enhanced by treatment with BDNF (100 ng/mL) for 1 hour. Treatment with DPI reduced the activity of NAD(P)H oxidase. The chemiluminescent signals are expressed as counts per minute per milligrams of protein. Data are expressed as medians, with 25th and 75th percentiles (boxes) and 10th and 90th percentiles (I bars). B, Dose response and time dependency of BDNF on NAD(P)H oxidase activity. In the dose-response study, CASMCs were stimulated with the indicated concentration of BDNF for 1 hour (left). In the time-course study, the cells were incubated for each period with 100 ng/mL BDNF (right). Graphs are representatives of 4 independent experiments. *$P<0.05$ for the chemiluminescent signals of CASMCs with and without BDNF preincubation. C, Effect of BDNF on reactive oxygen species generation in cultured human CASMCs. Generation of reactive oxygen species was assessed with the dihydroethidium method. Treatment with BDNF (100 ng/mL) increased superoxide generation vs control. Results are representative of 5 independent experiments.

studies indicated that NTs induce cell death in cerebral ischemia.[22] Thus, the intracellular signaling pathway mediated by NTs can act not only for survival but also as a proapoptotic or pronecrotic pathway in neuronal cells. Whether coronary BDNF induces cell necrosis or apoptosis of vascular cells warrants further investigation. BDNF enhanced the activity of NAD(P)H oxidase and the generation of superoxide in cultured smooth muscle cells. Because oxygen radicals activate matrix metalloproteinases,[23] the oxidative stress by BDNF might induce the instability of atherosclerotic plaques. On the other hand, BDNF has important roles in survival or as a development factor even in nonneuronal tissues such as endothelial cells.[24] BDNF in vasculature may work as a protective factor for endothelial cells in a counterregulatory mechanism. Further investigation is needed to clarify the precise mechanisms of BDNF in the pathogenesis of UAP.

In the present investigation, increased Cs-Ao differences in BDNF were observed in the UAP patents, although there was no significant difference in the numbers of diseased vessels or the degree of coronary stenosis between the SAP and UAP groups. These findings indicate that BDNF in the coronary circulation seems to influence the disease state of angina pectoris rather than the degree of coronary atherosclerosis and plaque formation. Acute coronary syndrome usually occurs at sites with <70% stenosis, as determined by angiographic studies performed in patients before the onset of coronary events. Therefore, BDNF might be involved in the vulnerability of atherosclerotic plaques. There are several possible origins of BDNF in the coronary circulation, including vascular smooth muscle cells, accumulating inflammatory cells, adventitial fibroblasts, cardiac myocytes, and neural cells. Recently, it has been reported that platelets release BDNF; therefore, activated platelets are a potential origin of BDNF.[25] However, the intense immunoreactivity in patients with UAP suggests that BDNF in the coronary circulation likely comes from atherosclerotic plaques. Plaque rupture and erosion are key events in the pathogenesis of acute coronary syndrome, including unstable angina; therefore, it is possible that disrupted atherosclerotic plaques release BDNF. Some other factors may be involved in the increased Cs-Ao differences in BDNF. Thus, further investigations are necessary to establish the causal relationship between BDNF and plaque rupture.

Psychological stress produces significant increases in heart rate and blood pressure, which might lead to an increased myocardial oxygen demand. Kario et al[26] demonstrated that earthquake-induced stress increased not only blood pressure and blood viscosity determinants but also fibrin turnover with endothelial cell stimulation in a group of hypertensive elderly subjects, suggesting that acute stress might trigger cardiovascular events. On the other hand, psychological stress increases the secretion of NTs from central and peripheral nerves.[27–29] Neuropsychological studies demonstrate that psychological stress such as immobilization stress increases BDNF mRNA expression in the hypothalamus of experimental models.[29] There is no direct evidence that BDNF in the coronary vasculature is regulated by psychological stress in

humans; however, psychological stress might increase the production of BDNF in coronary beds, which in turn augments regional oxidative stress via NAD(P)H oxidase. Further investigation is needed to clarify the regulation of coronary BDNF and the direct effect of psychological stress on NT levels in the coronary vasculature.

Plasma BDNF levels are decreased in patients with psychological disorders such as depression, and the level recovers with antidepressant drug treatment.[27,30] In our study, we did not enroll patients with mental disorders, and no patients had taken antidepressant drugs or tranquilizers. Although the psychological states of the patients were not examined, we do not consider that the Cs-Ao differences in BDNF were influenced by these factors.

In conclusion, plasma BDNF, but not NT-3, was increased in the coronary circulation in patients with UAP, and BDNF expression was enhanced in coronary arteries of UAP patients. BDNF increased NAD(P)H oxidase activity and superoxide production in human CASMC culture. Our observations suggest that the enhanced oxidative stress induced by BDNF has an important role in plaque instability.

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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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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.