緻密斑の細胞が感知して，なかでアデノシンが産生されます．産生されたアデノシンが腎臓の糸球体外メサンギウム細胞でアデノシン受容体に結合して細胞内カルシウムが上昇し，それがギャップジャンクションによって実際に輸入細動脈に伝わって収縮を起こすという経路がTGFの1つの経路です．

柏原——従来いわれたmacula densaにおけるnNOS由来のNOとアデノシンの両者が関与しているということですか．

林——そうですね．nNOSはいわゆるモジュレーターではないかと言われています．

柏原——輸出細動脈にも自動調節能があり，輸入細動脈と連動する形で，相反する方向に動くという先ほどのお話ですが，この協調的な動きの調節については何かデータは出ているのでしょうか．

林——アデノシンA2受容体ブロッカーを用いることによってブロックされるというデータがあります．アデノシンはA1受容体を刺激すると収縮して，A2受容体を刺激すると拡張するという特徴がありますので，同じアデノシンが出ている状況下で逆方向に動くということがあります．つまり輸入細動脈ではA1，輸出細動脈ではA2が関与している可能性があるのではないかと言われています．

柏原——TGFについても新しい知見が集積しつつありますので，次は各種の進行性腎障害における自動調節能の破綻の状態と，そのメカニズムについてご解説をお願いします．

林——何らかの原因で腎障害が起こると，輸入細動脈自体の灌流圧による収縮反応が見られます．これは慢性腎障害や糖尿病，インスリン抵抗性でや急性高血糖でも起こり得ることです．

糸球体の内圧が40～50mmHgに保たれているのが輸入細動脈における収縮のバランスですが，腎障害や糖尿病などで動脈硬化性病変が始まると一般的には血圧が上昇します．また，輸入細動脈の圧による収縮反応が減弱し，その結果，糸球体内圧が収縮反応の減弱によってさらに増加します．

したがって，治療方針として我々が行うべきことは，単に元のレベルまで血圧を下げただけでは糸球体内圧は上昇したままですから，糸球体内圧を正常に戻すため，元のレベルよりも更なる降圧が必要になります．

川嶋成乃亮 先生

内皮保護の観点から考える治療戦略としては，アンジオテンシンの阻害だけでなく，スタチンの抗酸化作用にも期待したい．

柏原——進行性腎障害には糸球体内における自動調節能の破綻を起因とした共通の病態が存在し，筋原性あるいはTGフィードバック障害があるということですが，細動脈レベルの動脈硬化性の腎障害，腎硬化症では糸球体内の血行動態にどのような異常が想定されていますか？

糸球体前血管に病変があり，虚血や組織学的にcollapseした糸球体もよく見られます．一方，それと共存する形で肥大した糸球体もよく見られますが，糸球体前血管，特に輸入細動脈レベルの血管障害がある場合，糸球体内の血行動態はどのように考えられますか．

林——5/6腎摘例で行ったデータを見ますと，micropunctureでは輸入細動脈の拡張が見られ，輸出細動脈の拡張は軽度で，そのため全身血圧が糸球体内圧に直接伝達されて最終的には糸球体高血圧，糸球体硬化をきたすといわれています．つまり糸球体が長期間にわたって高圧状態にさらされていることによって硬化をきたし，腎機能が低下する．腎機能低下によって残存糸球体がhyper functionを起こして，さらに悪化することは想

林　晃一 先生

腎障害による糸球体内圧の亢進を正常化するためには，元のレベルよりも更なる降圧が必要である．

定できると思います．

柏原——そのネフロン数減少モデルにおける糸球体内高血圧と共通した病態が，動脈硬化性の腎硬化症において認められると考えられるわけですね．

林——ヘテロジェネィティはやはり組織学的にもあると思いますし，function にもそういうふうに考えています．

2．各種薬剤の糸球体血行動態に対する作用

柏原——さて，次に薬物療法に話題を移したいと思います．進行性腎疾患には共通基盤があり，その血行動態を改善させるためにも降圧療法の重要性というのはもう十分証明されており，各種の降圧薬間には糸球体内の血行動態の異常を是正する作用に差があります．特にジヒドロピリジン系カルシウム（Ca）拮抗薬間には各々固有の効果があると報告されています．腎内の各種の Ca チャネルの分布，あるいは Ca 拮抗薬の血行動態の改善作用について林先生からご解説をお願いします．

林——従来の Ca 拮抗薬のプロトタイプというとニフェジピンですが，これは独占的にL型の電位依存性 Ca チャネルを抑制する薬剤だといわれています．これを実際に腎臓で作用させた我々のデータでは，アンジオテンシンIIで輸入細動脈が収縮したところへニフェジピンを投与すると，輸入細動脈だけが拡張して輸出は開かないということが見られます．つまりこれは，輸入にはL型が働いていて，輸出には働いていないということを示唆しています．

ただ，この作用が Ca 拮抗薬全般で認められるわけではなく，マニジピン，エフォニジピンでは輸入細動脈だけでなく輸出細動脈においても拡張作用を示します．Ca チャネルには特に電位依存性が強いL型があり，これは従来の Ca 拮抗薬の作用部位になります．最近，特に我が国で開発された Ca 拮抗薬には，L型のみならずT型にも作用する，あるいはN型にも作用することがわかってきました．

内科学会で発表された，帝京大学の古川先生がパッチクランプで行われたデータでは，従来のニフェジピンではL型に優先的に作用するのに比べ，エフォニジピンではT型，L型の両方に作用します．実際にT型により選択的なミベフラジルという薬剤を用いますと，アンジオテンシンIIによって収縮していた輸入細動脈，輸出細動脈で共に拡張が認められました．

また，N型に作用する薬剤で特に有名なのがシルニジピンですが，シルニジピンにおいても同じように輸入細動脈と輸出細動脈の拡張が認められています

柏原——従来からのジヒドロピリジン系の Ca 拮抗薬はL型 Ca チャネルを抑制して輸入を拡張する，T型あるいはN型の Ca チャネル抑制作用をもった Ca 拮抗薬については輸出細動脈の拡張作用も期待できるということで，腎臓にとってはより有効性が期待できるということですね．

基礎データから十分予測できる結果ですが，これを実際に証明した，Ca 拮抗薬間の非 class effect の差について調べた臨床データはございますか？

林——実際に利用できる臨床データはエフォニジピンがありますが，他の Ca 拮抗薬と比較したデータはありません．ACE 阻害薬とエフォニジピンと比較した我々の成績がありますが，ACE 阻害薬と同等にエフォニジピンによる尿蛋白の減少が

認められました．特にエフォニジピンの作用を，血圧が下がらなかった群と下がった群に分類して検討したところ，大幅に降圧しなかった群でも尿蛋白の減少が認められました．

柏原——従来，Ca 拮抗薬の腎保護作用は，厳格な降圧が達成できていないと発揮できないと言われていましたが，輸出細動脈の拡張能をもった Ca 拮抗薬については，厳格な降圧が達成できない状況下でも糸球体の血行動態を改善し得た可能性があるということですね．

Ⅲ 腎疾患における血管内皮機能障害の意義

柏原——最近，糸球体の病変の障害よりも，むしろ間質病変の障害度が最終的な腎機能予後と相関することが十分証明されてきたと思います．この間質尿細管障害のメカニズムにおいて，間質の虚血が重要だと示されていますが，そのご解説を南学先生にお願い致します．

南学——糸球体は毛細血管の集合体という構造ですから，この一連の微小血管において，その毛細血管の血管内圧というのは非常に重要な役割を果たしています．

血管の内皮細胞の働きというのは，①血栓予防作用，②炎症調節作用，③細胞外基質の産生作用などがありますが，当然これらの作用は糸球体の内皮細胞においても重要で，糸球体に障害が起こると，上記の①～③の作用に障害が起こります．

糸球体の内皮細胞研究は従来より動物モデルで主に行われ，まず最初に可逆性モデルで，糸球体障害から回復して元の糸球体に戻るときに糸球体の内皮細胞の増殖が見られるという知見が得られました．一方，糸球体硬化に至るモデルでは，糸球体内皮細胞のアポトーシスが見られるということも記載されています．

WKY ラットで抗 GBM 腎炎を起こした場合，糸球体内皮細胞にアポトーシスが起こり，その部分で硬化が起こること，あるいは古くは5/6腎摘で硬化に至る，その最初の変化として内皮細胞障害が起こるということが報告されています．

南学　正臣 先生

血管新生・血管保護の観点から，生体に備わる低酸素応答性の転写調節因子 HIF を利用した戦略に期待している．

柏原——動脈障害や動脈硬化の早期の共通した基盤として，レドックスの破綻も含めた内皮の機能異常が起こることを川嶋先生からご解説頂きましたが，糸球体というのは特殊な血管と考えるべきなのでしょうか？　他臓器の血管障害と共通した機序が存在するのでしょうか？

南学——糸球体の内皮細胞は窓が多数あいていて，濾過に非常に有利な構造をしているという点では特異的だと思いますが，障害腎の炎症や細胞外基質の産生機序は他の内皮細胞と共通だろうと私は考えています．

柏原——糖尿病の細小動脈障害においても，早期から内皮の機能異常が認められますが，糸球体の内皮の機能異常についてはあまり語られていませんよね．それは技術的な問題があるから，糸球体の内皮の機能を評価できないということなのでしょうか．

南学——おっしゃる通りだと思います．培養細胞に関してもいくつか細胞株はありますが，主流はヒト臍帯静脈由来血管内皮細胞（HUVEC）ですし，技術的な問題なのだと思います．

実際に内皮細胞障害を誘導する薬剤を投与して同時に抗 GBM 腎炎を誘導すると，糸球体硬化が促進するというスタディもありますので，やはり

内皮細胞障害は糸球体硬化の進展に非常に重要であると考えられます．逆に，内皮細胞が正しく増殖し障害を修復することが，糸球体障害から正常に戻るために重要だと考えられます．

柏原——次に，最近，糸球体から post glomerular capillary としての間質毛細血管障やその障害に起因する間質の重要性が示されていますが，そのご解説をお願い致します．

南学——腎不全というのは，一定の障害レベルに達すると，あとは final common pathway を経て末期腎不全に至るということが知られていますが，その1つとして尿細管間質の慢性虚血が重要であるということがわかってきました（**図2**）．

その機序ですが，尿細管間質へ酸素あるいは栄養を供給しているのは輸出細動脈の下流にある傍尿細管毛細血管で，傍尿細管毛細血管への血流低下あるいは傍尿細管毛細血管の障害によって尿細管間質への酸素供給が低下し，最終的に末期腎不全に至ることがわかっています（**表1**）．

糖尿病性腎症ラットを例にあげますが，デオキシヘモグロビンを測定する blood oxygen level dependent（BOLD）法による functional MRI 画像では，非常に早期から腎臓が虚血に陥っているということがわかっています．その理由としては，hyper filtration に伴って多量に濾過される尿細管内の液に対して再吸収を行うことによりエネルギー需要が増加し，相対的な虚血に陥ると考えられています．その他のモデル，例えば Dahl 食塩感受性高血圧ラットの尿細管間質の虚血やアンジオテンシンⅡ持続投与モデルにおける尿細管間質の虚血なども，柏原先生のグループを中心に研究が行われていますが，やはり障害進展に重要であると示されています．我々の自験例でも，Thy1 腎炎の片腎を摘出し，さらに Thy1 抗体を2回注射してこの腎炎モデルを慢性進行性にしたところ，尿細管間質の虚血状態が観察されました．やはり尿細管間質は非常に重要な部位であると思います．

柏原——その機序についてですが，一定以上の糸球体病変が生ずると，その下流の血管に血行障害が起きると考えてよろしいですか．

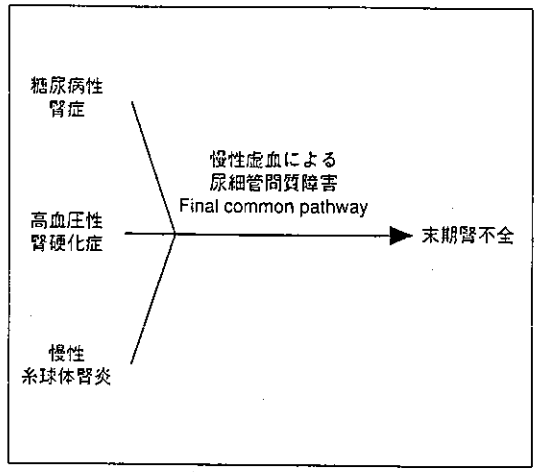

図2　腎不全進行の原因（慢性虚血）

表1　腎臓における組織の低酸素の原因

●傍尿細管毛細血管の喪失
●組織の線維化による傍尿細管毛細血管から尿細管・間質細胞への酸素の拡散能の低下
●糸球体硬化および血管作動性物質の imbalance による傍尿細管毛細血管の血流低下
●尿細管のエネルギー需要の増大（hyper-filtration, proteinuria など）による相対的酸素不足
●貧血による酸素の delivery 不足

南学——片腎 Thy1 慢性進行性腎炎モデルは早期から糸球体の血管が障害されるので，1つは傍尿細管毛細血管の上流にある糸球体が障害されることによって下流の血流が遮断されるのだろうと思います．

腎障害が進行した時期には尿細管間質が線維化しますので，そうなると，その領域に酸素は供給されなくなります．また，線維化に伴って尿細管細胞，あるいは間質細胞と傍尿細管毛細血管の距離が開くため酸素が拡散できなくなり低酸素に陥るというのが，更に進行した時期の低酸素の機序です（**表1**）．

一方，早期では，血管作動性物質の異常が考えられます．先ほどから話題に出ているアンジオテンシンⅡですが，これは輸出細動脈を選択的に収縮するので，輸出細動脈の下流にある傍尿細管毛細血管の血流も低下し，アンジオテンシンⅡ過剰

状態ではやはり尿細管間質が虚血に陥ります.

柏原――病変の進展度に応じてメカニズムも異なるということですね.

以上の話を踏まえて,治療への展望,間質の虚血を改善し得る治療戦略をご紹介頂けますか.

南学――まず腎不全の患者さんに対する治療戦略としては,腎性貧血の改善が臨床で応用されています.腎性貧血は早期から認められますので,貧血改善によってヘモグロビンが増加し,酸素供給量が増加する,これは非常に有効な手段だと考えています.また,最近の知見では,エリスロポエチン自体が血管内皮前駆細胞を誘導して血管新生を促進するデータも示されています.実際に末期腎不全患者さんにエリスロポエチンを投与したところ,末梢血中の血管内皮前駆細胞が増加したと報告されておりますので,エリスロポエチンの作用機序には腎性貧血の改善に加えて血管保護の役割もあるであろうと考えています.

血管新生や血管保護の観点から考えますと,当然 vascular endothelial growth factor (VEGF) など,成長因子による治療戦略が考えられます.これに関しても実験動物でいくつかの知見があり,例えばハブ毒と Thy1 を同時に投与して強度の腎炎を起こした場合に VEGF を投与すると,糸球体硬化が防げるというデータもありますし,5/6 腎摘モデルに VEGF を投与すると糸球体硬化が防げるというデータも示されています.

ただ,VEGF 投与による血管新生の誘導は,単独の成長因子によって行われるので,あまり生理的ではありません.どうしても透過性に異常があったり,構築に異常のある血管が新生する可能性もあるので,生体がもともと備えている低酸素応答性の転写調節因子 hypoxia inducible factor (HIF) を利用する最新の戦略を考えています.転写調節因子 HIF-1α と VP16 のハイブリッドを安定化させて遺伝子治療に用いる方法が循環器分野で既に行われており,実際に冠動脈疾患や閉塞性動脈硬化症に関しては米国で治験が始まっています.我々も腎疾患モデルに投与してみましたが,有効性を確認しています.

IV 動脈硬化性腎障害の治療戦略

柏原――進行性の腎障害,特に動脈硬化性の腎障害においても一般的な動脈硬化と重複する病態があり,治療についても同じ戦略で臨むことができるとわかってきましたが,それらを踏まえて,進行性腎障害でも今後の増加が予測される動脈硬化性腎障害,あるいは腎硬化症治療への考え方について各先生からご意見を伺いたいと思います.

川嶋――広い視野で考えた場合,やはり内皮保護が非常に重要なポイントになると思います.内皮保護にはアンジオテンシンのブロックだけでなく,スタチンも重要になるでしょう.スタチンの pleiotropic 作用,そのなかでも特にスタチンの抗酸化作用について,今後へ期待できる結果が出てくるのではないかなと思っています.SHR ラットにスタチンを投与して脳卒中への影響を調べた自験例で,腎臓への影響も同時に調べたところ,スタチンの投与による腎障害の軽減を見出しました.その機序としては,酸化ストレスの軽減が考えられましたので,このような戦略も成り立つのではないかなと思っています.

もう1つの考え方として,これは全くの仮説ですが,内皮の前駆細胞を利用した治療の可能性が考えられます.足の血管に前駆細胞を移植すると内皮機能の改善が認められます.この機序として,移植した細胞から様々な内皮を修復するような成長因子,サイトカインが出ているという考え方が1つと,移植した細胞自身が内皮のリニューアルに働いているのではないかという考えがあります.この考え方を取り入れると,下肢の血管だけでなく全身の血管の保護・修復にも有効になるのではないかと思っています.ただ,腎臓についてはまだ全く不明ですので,今後に期待したいと思います.

柏原――林先生,先ほど南学先生から間質の虚血の重要性についてお話し頂きましたが,レニン・アンジオテンシン (RA) 系阻害薬や Ca 拮抗薬と糸球体内及び間質の血行動態への有用性につい

てお話し頂けますか.

林——腎障害がある場合，一般的にはRA系が亢進しているので輸入・輸出細動脈あるいは直血管などの収縮が認められ，その観点から考えればARBやACE阻害薬は血流改善の有効な手段になると思います.

柏原——腎血流も増加しますから，間質の血流も増加すると考えてよろしいですか.

林——そうですね．血流増加とともにアンジオテンシンIIの直接作用を抑制する作用もあると思います.

柏原——輸出細動脈の拡張作用を持たないCa拮抗薬も輸入細動脈の拡張を介して腎血流を増加させるので，間質血流についても決して悪くないように思うのですが，いかがでしょうか.

林——Ca拮抗薬ではナトリウム利尿作用がありますが，この作用には輸出細動脈を拡張する作用によるものと輸入細動脈を拡張させる作用によるものがあります．ニフェジピンを代表とするL型Ca拮抗薬でも，ナトリウム利尿が起こることは臨床的にも認められていますので，髄質への血流増加があるといわれています.

柏原——それら薬剤を含めた動脈硬化性の腎硬化症に対する全般的な治療のあり方についてはいかがお考えですか.

林——一般的にはRA系降圧薬が推奨されていますが，皆様ご承知の通り，単剤での治療は実際に難しいわけです．薬剤を3～4剤併用して腎性高血圧は治せないという観点から考えると，Ca拮抗薬などとの併用療法が重要になると思いますね.

柏原——南学先生，間質の血流回復のための治療について，現時点で存在する治療手段をどのように組み合わせれば有効でしょうか？

南学——K/DOQIのガイドラインでも，動脈硬化に対する治療と重なっている部分が多いことがわかります．厳格な血糖と血圧の管理，脂肪を低下させるようスタチンを使用すること，さらに貧血改善や腎臓特異的な低蛋白食，ACE阻害薬やARBを中心とした降圧薬の使い方が記載されており，多面的にリスクを治療しなければいけないことがわかります.

腎疾患治療でゴールデンスタンダードとなっているACE阻害薬やARBには，腎臓の皮質の酸素化を改善するという作用も示されていますし，我々も5/6腎摘のモデルを使って傍尿細管毛細血管の血流がARBで改善することを示しました．また，柏原先生の実験で，Ca拮抗薬のアゼルニジピンを使うと尿細管間質の血流が改善するということも示されていますので，やはり輸出細動脈を拡張し，傍尿細管毛細血管の血流を増加させる作用のあるCa拮抗薬には腎保護作用があるのではないかと考えられますね.

柏原——動脈硬化性の腎硬化症は非常に増加傾向にあり，透析導入後の生命予後も決して良好ではない．その治療戦略の構築が急務ですが，その1つには，動脈硬化全般に共通するものとしてatherogenicなリスクへの多面的に介入が重要になるとまとめることができますね．特に腎臓については，糸球体内の血行動態の改善と間質虚血の改善という2つの視点から，血行動態を改善する考え方を構築する必要があるでしょう．今後，各種薬剤の合理的な組み合わせが構築されることを期待しております.

本日はどうもありがとうございました.

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The Two Faces of Endothelial Nitric Oxide Synthase in the Pathophysiology of Atherosclerosis

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In the endothelium, nitric oxide (NO) is constitutively generated from the conversion of L-arginine to L-citrullin by the enzymatic action of endothelial NO synthase (eNOS). An impairment of endothelium-dependent relaxation (EDR) is present in atherosclerotic vessels even before vascular structural changes occur, and represents the reduced eNOS-derived NO activity. Because of its multiple biological actions, NO from eNOS is believed to act as an anti-atherogenic molecule. On the other hand, there is increased production of superoxide in atherosclerotic vessels, which promotes atherogenesis. Recently it is revealed that eNOS becomes dysfunctional and produces superoxide rather than NO under various pathological conditions in which tissue levels of BH4 are reduced. The pathological role of dysfunctional eNOS has attracted attentions in vascular disorders including atherosclerosis, in which abnormal pteridine metabolisms in vascular tissue including decreased BH4 levels and increased BH2 levels have been demonstrated. The presence of dysfunctional eNOS may not only impair EDR but also accelerate lesion formation in atherosclerotic vessels. This review focuses on two faces of eNOS as both an NO- as well as superoxide-producing enzyme depending on tissue pteridine metabolisms in the pathophysiology of atherosclerosis.

Keywords Atherosclerosis, BH4, Endothelial Nitric Oxide Synthase, Nitric Oxide, Superoxide

Nitric oxide (NO) is generated from the conversion of L-arginine to L-citrullin by the enzymatic action of an NADPH-dependent NO synthase (NOS) (Moncada et al. 1991; Stuehr 1999). NOS consists of three isoforms, namely neuronal NOS (nNOS), inducible NOS, and endothelial NOS (eNOS). NOSs contain two catalytic domains that consist of a C-terminal reductase domain where NADPH, FMN, and FAD bind, and the N-terminal oxygenase domain where heme, 5,6,7,8-tetrahydrobiopterin (BH_4), oxygen, and L-arginine bind. The catalytic mechanisms of NOSs involve flavin-mediated electron transport from C-terminus–bound NADPH to the N-terminal heme center, where oxygen is reduced and incorporated into the guanidine group of L-arginine, giving rise to NO and L-citrulline. In vessels, NO is constitutively produced from the endothelium by the eNOS, which is activated by mechanical stress such as blood flow–mediated shear stress and stimulation with agonists such as bradykinin and acetylcholine. NO from the endothelium controls vascular tone, inhibits monocyte and leukocyte adhesion to the endothelium, inhibits platelet aggregation, and decreases endothelial permiability (Moncada and Higgs 1993; Nathan and Xie 1994; Palmer et al. 1987). Endothelial production of NO also inhibits vascular smooth muscle cell migration and proliferation. Therefore, eNOS-mediated production of NO plays a crucial role in control of vascular homeostasis.

On the other hand, in vitro biochemical studies demonstrated that NOSs themselves can produce superoxide under certain conditions (Alp and Channon 2003; Tiefenbacher 2001; Wever et al. 1998). In the presence of suboptimal concentrations of L-arginine and/or BH_4, activation of NOS leads to "uncoupling of NOS" and subsequent production of superoxide (Pou et al. 1992; Stroes et al. 1998; Vasquez-Vivar et al. 1998; Xia et al. 1998). In "uncoupled NOS," electrons flowing from the reductase domain to the heme are diverted to molecular oxygen rather than to L-arginine, and thereby production of superoxide occurs. The ability of NOS to produce superoxide was first demonstrated in nNOS and then extended to eNOS (Mayer and Werner 1995; Schmidt et al. 1992). In the recombinant bovine eNOS, the heme moiety is identified as the main source for superoxide production (Wever et al. 1997). In endothelial cells, a close link between cellular BH_4 levels and NO synthesis was demonstrated and it is revealed that an optimal concentration of BH_4 is essential for NO production by eNOS (Cosentino and Luscher 1999; Vasquez-Vivar et al. 2003).The precise role of BH_4 in the formation of NO is not completely understood, but it is postulated that BH_4 stabilizes NOS, facilitates its binding to L-arginine, and donates electrons to the ferrous-dioxygen

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complex in the oxygenase domain (Stores et al. 1997; Vasquez-Vivar et al. 2002b).

NO produced from the endothelium by eNOS has been studied extensively and it is widely recognized that NO from eNOS acts as a protective factor for vascular homeostasis. Superoxide produced by dysfunctional eNOS, however, counteracts the actions of NO and serves to impair vascular integrity (Cai and Harrison 2000). Recently it is revealed that eNOS may become dysfunctional under pathological conditions such as diabetes mellitus, hyperlipidemia, and atherosclerosis, and the role of dysfunctional eNOS in such vascular disorders has attracted attentions. This review focuses on the divergent role of eNOS in vascular disorders, particularly in atherosclerosis.

NO AS EDRF

Among a variety of functions of NO, the action as the endothelium-derived relaxant factor (EDRF) is most important for vascular function (Drexler 1997; Moncada and Higgs 1993). An impairment of endothelium-dependent relaxations (EDRs) is present in atherosclerotic vessels even before vascular structural changes ensue (Creager et al. 1990). Also risk factors for atherosclerosis, such as hypercholesterolemia, hypertension, diabetes, and smoking, are associated with impaired EDR (Avogaro et al. 1997; Vanhoutte and Boulanger 1995). The impaired EDR represents the reduced activity of eNOS-derived NO, which is determined by a balance between the synthesis and breakdown of NO. There are several reasons to believe that the synthesis of NO is reduced in vessels with atherosclerosis and hyperlipidemia. The underlining mechanisms of the reduced NO synthesis include the reduced activity and expression of eNOS, decreased sensitivity of vascular smooth muscle to NO, and increased degradation of NO by reaction with superoxide (Harrison 1997). There is controversy regarding the expression of eNOS at the vessel wall. The reduction of eNOS expression is shown in advanced atherosclerosis, possibly due to reduced transcription of eNOS mRNA caused by cytokines (Oemer et al. 1998). However, several studies in animal models with atherosclerosis including ours demonstrate the unchanged or rather augmented expression of eNOS, at least in early atherosclerosis, despite the presence of impaired EDR (Kanazawa et al. 1996).

The impaired enzymatic activity of eNOS seems to play a central role in the reduced NO synthesis in atherosclerosis and hyperlipidemia. Proatherogenic lipids, such as oxidized low-density lipoprotein (oxLDL) and lysophosphatidylcholine, inhibit signal transduction from receptor activation to eNOS activation (Hirata et al. 1991; Miwa et al. 1997). Hypercholesterolemic serum as well as LDL up-regulates caveolin abundance, augments caveolin-eNOS heterocomplex, and thereby attenuates NO production from the endothelial cells (Feron et al. 1999). Endogenous NOS inhibitors such as asymmetric dimethylarginine (ADMA) are also involved in the mechanisms of reduced EDR in atherosclerosis (Cooke 2000).

On the other hand, the accelerated degradation of NO by superoxide is another important mechanism of the impaired EDR (Drexler 1997; Ohara et al. 1993). Because of the extremely rapid reaction rate of interaction between NO and O_2^-, it is conceivable that there is always some O_2^- reacting with NO within cells and in the extracellular space. Under physiological conditions, endogenous antioxidant defenses minimize this interaction and maintain a minute balance between O_2^- and NO. This minute balance is altered in pathological conditions such as hyperlipidemia and atherosclerosis. It has been revealed that superoxide production from vessels is augmented in both humans and animal models with atherosclerosis (Mugge et al. 1991; Ohara et al. 1995; Rikitake et al. 2001). Animal models of hyperlipidemia and atherosclerosis demonstrate an excess vascular superoxide flux that is linked to reduced NO bioactivity. As an evidence for the involvement of superoxide in the impaired EDR in atherosclerotic vessels, the restoration of EDR by antioxidants such as vitamine C and superoxide dismutase has been shown (Beckman et al. 2001; Mugge et al 1991; Taddei et al 1998).

REDUCED NO BIOACTIVITY AND ATHEROGENESIS

Endothelial dysfunction as characterized by an impairment of EDR, and thereby reduced bioactivity of eNOS-derived NO, is closely linked to prognosis of atherosclerotic diseases (Quyyumi 2003). In patients with angiographically normal coronary arteries as well as those with coronary artery disease, coronary vascular endothelial dysfunction predicts a worse long-term outcome from coronary events such as acute myocardial infarction and unstable angina. It has been shown that coronary events are more frequent in patients with depressed coronary vasodilatory responses to acetylcholine and/or adenosine (Halcox et al. 2002). This finding is consistent regardless of the method used to assess the endothelial function (Chan et al. 2003). Although the endothelium secretes multiple factors other than NO, these clinical studies suggest that eNOS-derived NO serves to protect vessels from progression of atherosclerosis.

Multiple functions of NO revealed by studies in vitro strongly imply that NO produced by eNOS protects vessels from atherogenesis (De Caterina et al. 1995; Lloyd-Jones and Bloch 1996; Sarkar et al. 1996). In vivo studies directed to modify the production of NO support the concept that eNOS-derived NO acts as an antiatherogenic molecule. Although the exact mechanisms are still not well clarified, chronic treatment with L-arginine, a substrate for NOS, inhibits atherosclerotic lesion formation in animal models of atherosclerosis, such as diet-induced atherosclerosis models of rabbits and mice (Cooke et al. 1992; Aji et al. 1997). On the contrary, NOS inhibitors such as N^{ω}-nitro-L-arginine methyl ester (L-NAME) significantly accelerate atherosclerotic lesion development, suggesting that inhibition of endogenous NO synthesis facilitates the progression of atherosclerosis (Kauser et al. 2000).

Recently eNOS gene–deficient mice were used to clarify more directly the role of NO produced by eNOS on

atherogenesis. Knowles et al. (2000) first demonstrated that genetic lack of eNOS resulted in enhanced atherosclerosis in association with hypertension in apolipoprotein E (apoE)/eNOS double-knockout mice, which were produced by crossing apoE knockout (apoE-KO) mice with eNOS knockout (eNOS-KO) mice. They suggested that the elevation of blood pressure was responsible for the increases in the lesion size in these mice, because there was a positive correlation between blood pressure and size of atherosclerotic lesions in aortas. More recently their group reported that the hypertensive and atherogenic effects of eNOS deficiency in apoE-KO mice depend on the presence of endogenous sex hormones (Hodgin et al. 2002). Kuhlencordt et al. (2001) also reported that eNOS deficiency resulted in a marked increase in atherosclerotic lesion size in aortas by use of apoE/eNOS double-knockout mice. In their study, the accelerated atherosclerosis was associated with peripheral coronary atherosclerosis and aortic aneurysm formation, and they reported that these changes were not caused by hypertension, because blood pressure reduction by hydralazine did not restore them (Chen et al. 2001). Therefore, although the participation of hypertension and sex hormones remains to be further clarified, these reports indicated that the absence of endogenous eNOS-derived NO accelerates atherosclerosis.

INCREASED SUPEROXIDE PRODUCTION FROM ATHEROSCLEROTIC VESSELS

Increased superoxide production is an important feature of atherosclerotic vessels and contributes to the pathophysiology of atherosclerosis (Ohara et al. 1993; Hathaway et al. 2002). Reaction with superoxide reduces the bioavailability of NO, impairs vasomotor function, increases platelet aggregation, and augments monocyte and leukocyte adhesion to the endothelium. Superoxide and other reactive oxygen species are involved in modification of lipids, induce expression of proinflammatory genes, and increase cellular proliferation. Superoxide can also activate matrix metalloproteinases and produce apoptosis, which may contribute to instability of atherosclerotic lesions. Therefore superoxide can be regarded in general as a proatherogenic molecule (Harrison et al. 2003; Sorescu et al. 2002).

Superoxide is produced by a variety of enzymes including mitochondrial respiration, arachidonic acid pathway enzymes lipoxygenase and cyclooxygenase, cytochrome P450s, NOSs, xanthine oxidase, and NAD(P)H oxidase (Cai and harrison 2000; Griendling et al. 2000). Among them, xanthine oxidase, NAD(P)H oxidase, and NOSs have been extensively studied in vasculature. Particularly, NAD(P)H oxidase plays a major role in vascular cells (Inoue et al. 1998; Sorescu et al. 2002). In atherosclerotic vessels, increased expressions of subcomponents of NAD(P)H oxidase have been revealed (Azumi et al. 1999, 2002). In the early stage of atherosclerosis, superoxide seems to be produced from NAD(P)H oxidase localized in the endothelium, and in the advanced atherosclerosis vascular smooth muscle cells serve as the major source of NAD(P)H oxidase–derived superoxide (Warnholtz et al. 1999).

NOS is also an important enzyme capable of producing superoxide. Recently it is revealed that superoxide is produced in vivo from dysfunctional eNOS under certain pathological conditions (Katusic 2001; Tiefenbacher 2001; Wever et al. 1998). Clinical as well as experimental studies demonstrated that acute administration of BH_4 improves endothelial dysfunction associated with hypercholesterolemia, atherosclerosis, hypertension, and cigarette smoking (Heitzer et al. 2000; Setoguchi et al. 2001; Stores et al. 1997; Tiefenbacher et al. 2000). These data have been used as an evidence for the presence of "uncoupled eNOS," which produces superoxide leading to impaired EDR, although the effect of exogenous BH_4 may be simply due to its potent direct antioxidant action. Also these studies suggest that BH_4 plays the crucial role in determining eNOS function in vivo and thereby in the pathogenesis of endothelial dysfunction. Regarding atherosclerotic vessels, Laursen et al. (2001) clearly demonstrated the production of superoxide from eNOS. In apoE-KO mice, they showed increased vascular superoxide production from the endothelium, which was associated with impaired EDR. Exposure of vessels from apoE-KO mice to sepiapterin, a precursor to BH_4, for 1 h improved EDR and decreased superoxide production, whereas sepiapterin had no effects on vessels from control mice (Laursen et al. 2001). Chronic BH_4 supplementation is also effective in restoration of endothelial dysfunction. Shinozaki et al. (2000) reported that oral supplementation for 8 weeks with BH_4 increased eNOS activity and reduced superoxide anion formation by eNOS in the aortas of insulin-resistant rats. Therefore it is conceivable that there is abnormality of BH_4 metabolisms, particularly reduced BH_4 availability, in the vascular tissue of atherosclerotic arteries.

VASCULAR PTERIDINE METABOLISM IN ATHEROSCLEROSIS

In normal vascular tissue, more than 60% of total BH_4 is present in the endothelium (Katusic 2001; Tsutsui et al. 1996). Only limited information is available on BH_4 contents and the pteridine metabolism in the vessel wall of the diseased states (Table 1). In diabetes mellitus, a reduction of BH_4 contents in vessel wall has been demonstrated. Endothelial cells from diabetic BioBreeding (BB) rats represent a marked reduction in BH_4 contents (Meininger et al. 2000). In the insulin resistance model of rats induced by high-fructose diet, a modest reduction of BH_4 levels in the aortas was associated with impaired EDR (Shinozaki et al. 1999). Furthermore, as compared with control rats, the levels of 7,8-dihydrobiopterin and biopterin, the oxidized forms of BH_4, were increased in the aortas of diabetic rats. The reduced BH_4 contents in vascular tissue are also reported in hypertension. In mice with deoxycorticosterone acetate (DOCA)-salt hypertension, BH_4 content was reduced and the content of oxidized forms of BH_4 was increased in aortas (Landmesser et al. 2003).

Regarding hyperlipidemia and atherosclerosis, Vasquez-Vivar et al. (2002a) reported that BH_4 levels in the aortas from diet-induced hypercholesterolemic rabbits were markedly reduced

TABLE 1
Vascular BH_4 levels in vascular disorders

Diseases	Models	Vascular BH_4 levels	References
Hypertension	DOCA-salt rats	Decreased	Landmesser et al. 2003
	Prehypertensive SHR	Unchanged	Cosentino and Luscher 1999
Diabetes	Diabetic BioBreeding rats	Decreased	Meininger et al. 2000
	Streptozotocin-induced Diabetes rats	Decreased	Alp et al. 2003
	Insulin-resistant rats	Decreased	Shinozaki et al. 2000
Atherosclerosis	Diet-induced hypercholesterolemic rabbits	Decreased	Vasquez-Vivar et al. (2002a)
	Apo-E-KO mice		
	with severe hypercholesterolemia	Decreased	Ozaki et al. 2002
	with mild hypercholesterolemia	Increased	d'Uscio et al. 2001
Postmenopausal	Ovariectomized rats	Decreased	Lam et al.

SHR: spontaneously hypertensive rat.

compared with those from normocholesterolemic rabbits. We also demonstrated that BH_4 levels in aortas were approximately 50% decreased in apoE-KO mice with marked hyperlchoesterolemia compared with normocholesterolemic wild-type mice (Ozaki et al. 2002). In contrast, d'Uscio et al. (2003) reported increased BH_4 levels in aortas of apoE-KO mice. The tissue levels of BH_4 are determined by the balance between its production and degradation (Alp and Channon 2003; Katusic 2001). Degradation of BH_4 by oxidation to 7,8-dihydrobiopterin is an important determinant of tissue BH_4 levels. BH_4 is a molecular target for oxidative stress and can rapidly be oxidized by reactive oxygen species such as peroxynitrite (Kuzkaya et al. 2003; Landmesser et al. 2003; Zou et al. 2002). In the study of Laursen et al. (2001), administration of exogenous peroxynitrite increased superoxide production from normal mouse aortas by an eNOS-dependent manner, suggesting that peroxynitrite oxidized BH_4 and led to uncoupling of eNOS. It is shown that oxidation of BH_4 is enhanced and vascular tissue levels of 7,8-dihydrobiopterin increase under the elevated oxidative stress. In hyperlipidemia and atherosclerosis, it is very likely that oxidation of BH_4 to 7,8- dihydrobiopterin is enhanced, because these conditions are associated with increased oxidative stress. The discrepant reports on the tissue levels BH_4 in atherosclerotic vessels may be related to the difference in oxidative stress.

The reduced synthesis can also be involved in the reduced BH_4 contents. BH_4 is synthesized from guanosine triphosphate (GTP) via a de novo pathway by the rate-limiting enzyme GTP cyclohydrolase I (GTPCH I). Alternatively the synthesis of BH_4 can occur via a socalled salvage pathway, which uses BH_2 as a substrate (Alp and Channon 2003). Therefore, the reduced activity or expression of GTPCH I results in the decreased BH_4 levels in tissue. Insulin stimulates BH_4 synthesis via activation of GTPCH I, and Shinozaki et al. (2000) reported that GTPCH I activity in the aorta was significantly lower in the insulin-resistance rats than that of control rats. On the other hand, at present no information is available on the enzymatic activity of GTPCH I in atherosclerotic vessels.

It is suggested that a very minute balance between BH_4 levels and eNOS determines the extent of eNOS activation and thereby the rates of NO production versus superoxide formation from eNOS (Alp et al. 2003). Only the completely reduced form of biopterine (BH_4) maintains NOS coupling of NADPH oxidation to NO synthesis, and it is proposed that the ratio of BH_4/7,8-dihydrobiopterin is important in determination of "uncoupling" of eNOS in addition to the absolute value of BH_4 (Vasquez-Vivar 2002b). Therefore oxidative stress causes "uncoupling" of eNOS not only by decreasing BH_4 levels but also by increasing the ratio of BH_4/7,8-dihydrobiopterin. Then, generation of superoxide and peroxinitrite from dysfunctional (uncoupled) eNOS induces a further reduction of BH_4 availability (Landmesser et al. 2003; Laursen et al. 2001).

DYSFUNCTIONAL eNOS AND ATHEROGENESIS

As described earlier in this paper, a deficiency of eNOS gene was shown to accelerate atherogenesis in apoE-KO mice (Hodgin et al. 2002; Knowles et al. 2000). However, a controversy may exist regarding the effects of eNOS gene deficiency on atherosclerotic lesion formation. In the model of high-cholesterol diet–induced atherosclerosis, Shi et al. (2002) demonstrated the paradoxical reduction of atherosclerotic lesion in eNOS-KO mice compared with wild-type mice. In their report, L-NAME, the NOS inhibitor, reduced LDL oxidation by endothelial cells obtained from wild-type mice but not those from eNOS-KO mice. They speculated that eNOS may contribute to the oxidation of LDL under the circumstance of hypercholesterolemia, and that the absence of eNOS-mediated LDL oxidation may lead to the reduction of atherosclerotic lesion formation in eNOS-KO mice. It is very likely that superoxide from the dysfunctional eNOS is involved in the mechanisms of eNOS-mediated LDL oxidation. Therefore it seems possible that dysfunctional eNOS accelerates atherogenesis under certain conditions such as hypercholesterolemia. We have examined the effects of eNOS overexpression on atherosclerotic

lesion formation with the use of transgenic mice (eNOS-Tg) that overexpress eNOS mainly in the endothelium (Amano et al. 2003; Kawashima et al. 2001; Ohashi et al. 1998). We crossed eNOS-Tg with apoE-KO mice and fed a "high-cholesterol diet." Unexpectedly, the atherosclerotic lesion areas were significantly larger in eNOS-overexpressing apoE-KO (apoE-KO/eNOS-Tg) compared with control apoE-KO mice (Ozaki et al. 2002). After 8 weeks on a high-cholesterol diet, the atherosclerotic lesion areas in the aortic sinus were increased by more than twofold in apoE-KO/eNOS-Tg mice compared with apoE-KO mice. Also, aortic tree lesion areas were approximately 50% larger in apoE-KO/eNOS-Tg mice after 12 weeks on a high-cholesterol diet. In apoE-KO/eNOS-Tg mice, we revealed the presence of eNOS dysfunction, demonstrated by lower NO production relative to eNOS protein levels and enhanced superoxide production in the endothelium. We also found decreased BH_4 and increased 7,8-dihydrobiopterin levels in aortas of apoE-KO/eNOS-Tg mice. Chronic supplementation with exogenous BH_4 reduced the atherosclerotic lesion size in apoE-KO/eNOS-Tg mice to the level comparable to apoE-KO mice, possibly through the improvement of eNOS function. Therefore chronic overexpression of eNOS does not inhibit, but accelerates atherosclerosis under hypercholesterolemia. In contrast, van Haparen et al. (2002) also cross-bred apoE-KO mice with another line of eNOS transgenic mice that they created, and reported that atherosclerotic lesion size was reduced by eNOS overexpression. As for the mechanisms, they cited the reductions of blood pressure and plasma cholesterol levels, although the mechanisms of reduced plasma cholesterol levels were not identified. The discrepancy between their study and ours may be explained at least partly by a difference in the balance between NO and superoxide production from the endothelium, which is likely due to the difference in the extent of oxidative stress. Certain pathological conditions such as severe hypercholesterolemia are associated with increased oxidative stress and the minute balance between eNOS protein levels and tissue pteridine metabolism are lost, resulting in eNOS uncoupling. It is tempting to suggest that the marked increase in superoxide in association with decreased NO production would promote atherogenesis under such conditions, although the role of eNOS dysfunction on atherogenesis needs further studies.

POSSIBLE ROLE OF DYSFUNCTIONAL eNOS IN VASCULAR REMODELING

Increasing evidence demonstrates the presence of eNOS dysfunction in hyperlipidemia and atherosclerosis, which may contribute not only to endothelial dysfunction but also to atherosclerotic lesion formation. Then there raises a question as to whether dysfunctional eNOS involves vascular structural disorders other than atherosclerosis. The cellular mechanisms of vascular remodeling in large vessels are closely similar to those of atherosclerosis and endothelial dysfunction plays a crucial role. We previously reported that eNOS overexpression inhibits lesion formation in a mouse model of vascular remodeling, where the common carotid artery was ligated just proximal to the carotid bifurcation and the carotid artery proximal to the ligation site was histologically examined at 4 weeks later (Kawashima et al. 2001). In this vascular remodeling model, the endothelium remains uninjured, but neointimal and medial thickening occurs in combination with a reduction in vascular diameter at the proximal portion of the ligation. We revealed that neointimal and medial areas were significantly reduced in association with diminished leukocyte infiltration in eNOS-Tg mice compared with wild-type mice, showing the protective role of NO from eNOS on vascular remodeling. Then we performed the study of a similar protocol in eNOS overexpressing apoE-KO mice (apoE-KO/eNOS-Tg). We found that the neointimal area was significantly increased by approximately 70% in apoE-KO/eNOS-Tg compared with that in apoE-KO mice. The increased neointimal lesion formation was associated with augmented infiltration of inflammatory cells and with elevated superoxide production from endothelial cells (Shinohara et al. 2004). Therefore it is very likely that dysfunctional eNOS contributes to the accerelated vascular remodeling under hypercholesterolemia in this model. It waits for the future studies to determine whether eNOS dysfunction may augment vascular remodeling under pathoplogical conditions with increased oxidative stress other than hypercholesterolemia, such as diabetes mellitus and hypertension.

TWO FACES OF eNOS IN VASCULAR DISORDERS

Endothelial NOS can be regarded as both an NO— as well as superoxide-producing enzyme and, therefore, eNOS may have dual effects on vascular function depending the vascular pteridine metabolisms. Under pathological conditions where pteridine metabolisms are impaired, eNOS may serve as an injury molecule by producing superoxide rather than NO (Figure 1). Although no studies are available, it is intriguing to speculate that, in addition to vascular structural disorders such as atherosclerosis and vascular remodeling, dysfunctional eNOS is involved in vascular functional disorders, including susceptibility to ischemia and impaired angiogenesis, that are present in pathological conditions.

The restoration of eNOS function in the diseased states by improving pteridine metabolisms may serve as a strategy for treatment of vascular disorders including atherosclerosis. We found that supplementation with exogenous BH_4 inhibits atherosclerotic lesion formation in apoE-KO mice (Ozaki et al. 2002). Although the detailed mechanisms are unclear, it is conceivable that, in addition to the simple removal of superoxide by its antioxidant effect, exogenous BH_4 improved pteridine metabolism at the vessel wall and led to restore normal eNOS function. On the other hand, GTPCH can be a rational target to augment endothelial BH_4 and normalize eNOS activity in endothelial dysfunction. Zheng et al. (2003) performed ex vivo gene transfer of human GTPCH I to carotid arteries of DOCA-salt rats and revealed an improvement of EDR accompanied by increased tissue BH_4 levels. Recently Alp et al. (2003) generated transgenic mice overexpressing GTPCH solely in the endothelium. They

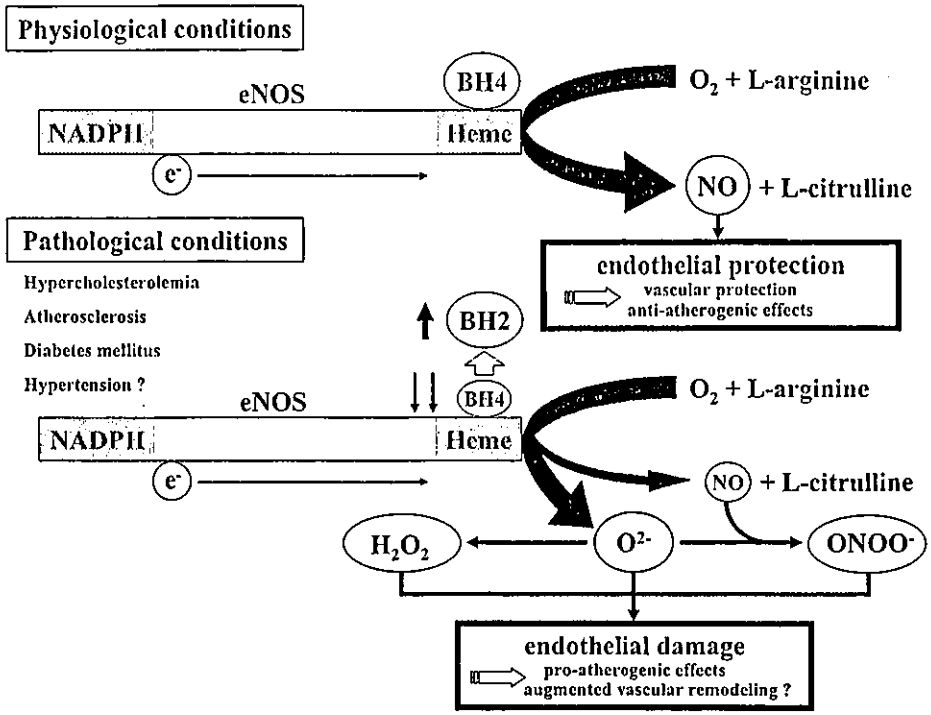

FIG. 1. Under physiological conditions, the minute balance between eNOS protein levels and tissue levels of BH_4 are maintained, and activation of eNOS generates NO and L-citrulline. NO generated by eNOS serves to maintain vascular integrity, and thereby inhibits atherogenesis and vascular remodeling. Under pathological conditions such as atherosclerosis and hyperlipidemia, where oxidative stress is increased, vascular BH_4 levels are reduced and BH_2 levels increased. Activation of eNOS leads to "uncoupling of NOS" with subsequent generation of superoxide rather than NO. Superoxide, and subsequently peroxinitrite and hydrogen peroxide, impair endothelial function, and may promote atherosclerogenesis and vascular remodeling.

reported that overexpression of GTPCH augmented endothelial BH_4 levels, improved the impaired vascular function, and decreased superoxide production from vessels in streptozotocin-induced diabetes. They suggested that a small increase in BH_4 levels in tissue was sufficient to maintain normal eNOS function (Alp et al. 2003). Very recently their group reported that crossing the GTPCH transgenic mice with apoE-KO mice resulted in a reduction in atherosclerotic lesions (Alp and Channon 2003).

On the other hand, simply augmenting NOS protein levels under pathological conditions such as hypercholesterolemia may not increase NO but augment superoxide production, resulting in detrimental rather than beneficial effects. Therefore, a strategy toward increasing NOS protein levels in association with maintaining its enzymatic activity is needed (Hattori et al. 2003).

CONCLUSION

Recent studies have revealed that eNOS becomes dysfunctional and produces superoxide rather than NO under pathological conditions such as diabetes mellitus, hyperlipidemia, and atherosclerosis. Dysfunctional eNOS is closely implicated in the endothelial dysfunction represented by impaired EDR in those vascular disorders. Although NO produced by eNOS with normal function acts as a protective or maintenance factor for vascular integrity, superoxide from dysfunctional eNOS may impair vascular function and structure. Under severe hypercholesterolemia, dysfunctional eNOS possibly promotes atherosclerotic lesion formation and augment vascular remodeling. For development of eNOS dysfunction, abnormality in BH_4 metabolism in vascular tissue seems to be fundamental, and therefore further understanding of tissue BH_4 metabolisms in diseased states is needed to develop therapeutic strategy based on eNOS function toward vascular disorders.

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Dysfunction of Endothelial Nitric Oxide Synthase and Atherosclerosis

Seinosuke Kawashima, Mitsuhiro Yokoyama

Abstract—Atherosclerosis is associated with an impairment of endothelium-dependent relaxations, which represents the reduced bioavailability of nitric oxide (NO) produced from endothelial NO synthase (eNOS). Among various mechanisms implicated in the impaired EDR in atherosclerosis, superoxide generated from dysfunctional eNOS has attracted attention. Under conditions in which vascular tissue levels of tetrahydrobiopterin (BH4), a cofactor for NOS, are deficient or lacking, eNOS becomes dysfunctional and produces superoxide rather than NO. Experimental studies in vitro have revealed that NO from eNOS constitutes an anti-atherogenic molecule. A deficiency of eNOS was demonstrated to accelerate atherosclerotic lesion formation in eNOS knockout mice. In contrast, eNOS overexpression with hypercholesterolemia may promote atherogenesis via increased superoxide generation from dysfunctional eNOS. Thus, eNOS may have 2 faces in the pathophysiology of atherosclerosis depending on tissue BH4 metabolisms. An improved understanding of tissue BH4 metabolisms in atherosclerotic vessels is needed, which would help in developing new strategies for the inhibition and treatment of atherosclerosis. (*Arterioscler Thromb Vasc Biol.* 2004;24:998-1005.)

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

Nitric oxide (NO) is generated from the conversion of L-arginine to L-citrulline by the enzymatic action of an NADPH-dependent NO synthase (NOS), which requires Ca^{2+}/calmodulin, FAD, FMN, and tetrahydrobiopterin (BH4) as the cofactors.[1-4] In the vessels, NO is produced from the endothelium by constitutive expression of the endothelial isoform of NOS (eNOS), which is activated by mechanical stress such as blood shear-stress and stimulation with agonists such as bradykinin and acetylcholine. NO has a variety of functions, but its action as the endothelium-derived relaxing factor (EDRF) is the most important for the maintenance of vascular homeostasis.[5] An impairment of endothelium-dependent relaxations (EDR) is present in atherosclerotic vessels even before vascular structural changes occur and represents the reduced eNOS-derived NO bioavailability. Endothelial dysfunction as characterized by an impairment of EDR, and thereby reduced eNOS-derived NO bioactivity, is the critical step for atherogenesis. Among various mechanisms responsible for the impaired EDR, the increased NO breakdown by superoxide is important, and there is augmented production of superoxide in atherosclerotic vessels. Recently, it was revealed that under certain circumstances, eNOS becomes dysfunctional and produces superoxide rather than NO. The pathophysiological role of dysfunctional eNOS has attracted attentions in vascular disorders, including atherosclerosis. This review focuses on the role of dysfunctional eNOS on atherosclerotic vessels and refers to the possible role of dysfunctional eNOS on atherogenesis.

Impaired EDR in Atherosclerosis

All major risk factors for atherosclerosis such as hyperlipidemia, diabetes, hypertension, and smoking are associated with impaired EDR.[6-8] Although the underlining mechanisms of the reduced EDR are multifactorial, its most important cause is a derangements of the eNOS/NO pathway, which include the reduced activity and expression of eNOS, decreased sensitivity to NO, and increased degradation of NO by reaction with superoxide.[8] Regarding the expression of eNOS at the vessel wall, it may be reduced in advanced atherosclerosis, possibly because of reduced transcription and/or increased instability of eNOS mRNA caused by cytokines.[9] However, most animal models with atherosclerosis demonstrate the unchanged or rather augmented expression of eNOS, at least in early atherosclerosis, despite the presence of impaired EDR.[10,11]

The enzymatic activity of eNOS is inhibited by various mechanisms associated with atherosclerosis and hyperlipidemia. Pro-atherogenic lipids, such as oxidized low-density lipoprotein (oxLDL) and lysophosphatidylcholine, inhibit signal transduction from receptor activation to eNOS activation.[12-14] Hypercholesterolemic serum and LDL upregulate caveolin abundance, augments caveolin–eNOS heterocomplex, and thereby attenuates NO production from the endothelial cells.[15,16] Endogenous NOS inhibitors such as asymmetric dimethylarginine (ADMA) and N-monometylarginine (NMA) are also revealed to be involved in the mechanisms of reduced EDR in atherosclerosis.[17,18]

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The accelerated degradation of NO by increased superoxide from vessel wall is demonstrated as another important mechanism of the reduced EDR in hyperlipidemia and atherosclerosis.[8] Superoxide production from atherosclerotic vessels is augmented in human and animal models with atherosclerosis.[19-22] The endothelium is important as a source of superoxide production, and its denudation decreases superoxide production from vessels with atherosclerosis but has no effects in normal vessels without atherosclerosis.[19] Animal models of hyperlipidemia and atherosclerosis demonstrate an excess vascular superoxide flux that is linked to reduced NO bioactivity. As an evidence for the involvement of superoxide in the impaired EDR in atherosclerotic vessels, the restoration of EDR by antioxidants and superoxide dismutase has been shown.[20,23,24] In rabbit aortas with high-cholesterol diet-induced atherosclerosis, the impaired vasodilatory responses to acetylcholine and A23187 were restored by chronic treatment with polyethylene-glycolated SOD.[20] Antioxidants improve EDR in human and animal models with atherosclerosis.[25-27] In particular, vitamin C is effective in the restoration of EDR associated with most risk factors for atherosclerosis, including hypercholesterolemia, hypertension, diabetes mellitus, and smoking.[28-30]

Superoxide Production From Vessels

Superoxide is produced by a variety of enzymes, including xanthine oxidase, cyclooxygenase, and NADPH oxidase. Among them, NADPH oxidase plays a major role in vascular cells.[31,32] In normal vessels, NADPH oxidase is present in adventitial fibroblasts. In atherosclerotic vessels, increased expression of subcomponents of NADPH oxidase has been found.[33-36] In the early stage of atherosclerosis, superoxide seems to be produced from NADPH oxidase localized in the endothelium; in advanced atherosclerosis, vascular smooth muscle cells serve as the major source of NADPH oxidase-derived superoxide.[37]

However, in vitro biochemical studies demonstrated that NOS can independently produce superoxide under certain conditions.[38-41] The catalytic mechanisms of NOS involve flavin-mediated electron transport from C-terminal–bound NADPH to the N-terminal heme center, where oxygen is reduced and incorporated into the guanidine group of L-arginine, giving rise to NO and L-citrulline. The eNOS-mediated superoxide generation is primarily regulated by BH4 availability. In the presence of suboptimal concentrations of BH4, activation of NOS leads to "uncoupling of NOS" and subsequent production of superoxide.[42-45] In "uncoupled NOS," electrons flowing from the reductase domain to the heme are diverted to molecular oxygen rather than to L-arginine; thereby, production of superoxide occurs. The ability of NOS to produce superoxide was first demonstrated in neuronal NOS (nNOS) and then extended to eNOS.[46,47] In the recombinant bovine eNOS, the heme moiety was identified as the main source for superoxide production.[45] In endothelial cells, a close link between cellular BH4 levels and NO synthesis was demonstrated, suggesting that an optimal concentration of BH4 is essential for NO production. The precise role of BH4 in the formation of NO is not completely understood, but it is postulated that BH4 donates electrons from the reductase domain to the ferrous–dioxygen complex in the oxygenase domain.[48,49] It is also demonstrated that addition of exogenous BH4 increases NO production and decreases superoxide production from endothelial cells.[40] As mentioned later in this article, there is an interaction between NADPH oxidase and eNOS, and it is thought that superoxide produced by NADPH is involved in the uncoupling of eNOS.

Exogenous BH4 and eNOS Function

It has been demonstrated in clinical and animal studies that acute administration of BH4 improves endothelial dysfunction associated with hypercholesterolemia, atherosclerosis, hypertension, and cigarette smoking.[50-53] These data have been presented as evidence for the presence of "uncoupled eNOS," which produces superoxide rather than NO, leading to impaired EDR. Laursen et al clearly demonstrated the production of superoxide from eNOS.[54] In apolipoprotein E-knockout (apoE-KO) mice, they showed the increased vascular superoxide production from the endothelium, which was associated with impaired EDR. Incubation of vessels with sepiapterin, a precursor to BH4, improved EDR and decreased superoxide production.

As in the study of Laursen et al, sepiapterin has been shown to restore endothelial function in acute studies, however, sepiapterin may not always be effective when vessels are exposed to it for a long time.[55-57] Sepiapterin is an oxidized BH4 analogue that generates BH4 by enzymatic reduction of sepiapterin reductase and dihydrofolate reductase. It is reported that relatively long-term (6 hours) incubation of hyperlipidemic rabbit vessels with sepiapterin resulted in a further derangement of vasodilatory response to endothelium-dependent agonists.[58] In addition, incubation of canine cerebral arteries with high levels of sepiapterin was shown to reduce EDR significantly, despite an increase in vascular BH4 levels. It is revealed that a high concentration of sepiapterin can serve as a pro-oxidant and thereby oxidizes BH4 to dihydrobiopterin (BH2).[49] Sepiapterin may increase BH2 rather than BH4 in the tissues, and the increased BH4 levels potentially compete with BH4 for eNOS binding and worsen eNOS uncoupling.

Vascular Pteridine Metabolism in Atherosclerosis

The presence of eNOS dysfunction as a mechanism of impaired endothelial function seems to be well-recognized now. However, only limited information is available on pteridine metabolism in the vessel wall in diseased states. In normal vascular tissue, >60% of total BH4 is present in the endothelium.[38,59] Endothelial cells from diabetic BioBreeding (BB) rats have a marked reduction in BH4 contents.[59] In the insulin resistance rat model induced by high-fructose diet, a modest reduction of BH4 levels in the aortas was associated with impaired EDR.[60] Furthermore, as compared with control rats, the levels of 7,8-dihydrobiopterin and biopterin, the oxidized form of BH4, were increased in the aortas of diabetic BB rats. Plasma BH4 levels were decreased in SHR with established hypertension.[61] Recently, it was reported that BH4 content was reduced and the content of oxidized forms of BH4 was increased in vessels from mice with deoxycorticosterone (DOCA)-salt hypertension.[62]

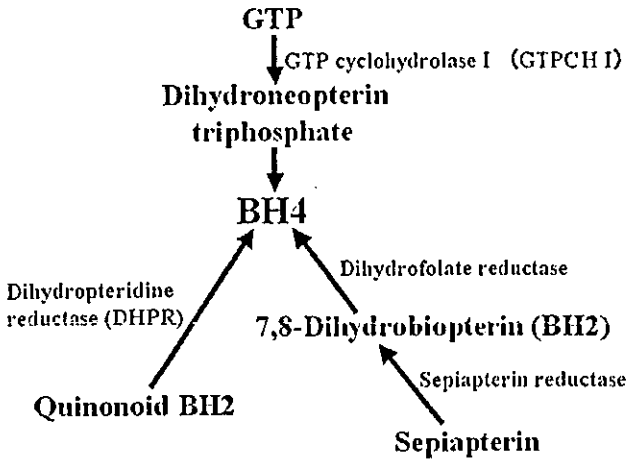

Figure 1. Scheme of BH4 biosynthesis.

Regarding hyperlipidemia and atherosclerosis, Vasquez-Vivar et al reported that BH4 levels in the aortas from diet-induced hypercholesterolemic rabbits were markedly reduced compared with those from normocholesterolemic rabbits.[58] We have also demonstrated the BH4 levels in the aortas were decreased ≈50% in apoE-KO mice with marked hypercholesterolemia compared with normocholesterolemic wild-type mice.[63] In contrast, d'Uscio et al reported that in the aortas of apoE-KO mice with moderate hypercholesterolemia, BH4 levels were increased by ≈1.8-fold compared with those in control mice.[64]

The tissue levels of BH4 are determined by a balance between its production and degradation. As shown in Figure 1, BH4 is synthesized from GTP via a *de novo* pathway by the rate-limiting enzyme guanosine 5′-triphosphate (GTP) cyclohydrolase I (GTPCH I). Alternatively, the synthesis of BH4 can occur via a so-called salvage pathway, which uses BH2 as a substrate. Therefore, the reduced activity or expression of GTPCH I results in the decreased BH4 levels in the tissue. In the insulin resistance rat model, Shinozaki et al reported that GTPCH I activity in the aorta was significantly lower than that of control rats.[65] We also found the reduced vascular GTPCH I activity in apoE-KO mice fed a "high-cholesterol diet" (S Kawashima et al, article under submission). Although the activity of GTPCH I is augmented by inflammatory cytokines such as TNF-α and IL-1β, which are activated in atherosclerotic vessels, GTPCH I gene expression is reduced by oxidized LDL.[66–68] The mechanisms of the reduced GTPCH I activity in the aortas of apoE-KO mice are currently under investigation. However, the tissue levels of BH4 are also determined by their gradation, namely by their oxidation to 7,8-dihydrobiopterin.[38] Studies in vitro showed that BH4 can be rapidly oxidized by reactive oxygen species such as peroxynitrite.[62,69] In DOCA-salt hypertensive mice, it was demonstrated that superoxide produced by NADPH oxidase led to the formation of peroxynitrite in reaction with NO, which induced uncoupling of eNOS. With elevated oxidative stress, the oxidation of BH4 is enhanced and vascular tissue levels of 7,8-dihydrobiopterin increase. Therefore, the discrepant results in vascular BH4 levels in hyperlipidemia and atherosclerosis can be at least partly explained as caused by the difference in the levels of oxidative stress. The studies of Vasquez-Vivar et al and ours were conducted in animals with severe hypercholesterolemia, which is likely associated with high oxidative stress, and d'Uscio et al used animals with mild hypercholesterolemia.[58,63,64]

It has been proposed that in addition to the absolute availability of BH4, the ratio of BH4/7,8-dihydrobiopterin, the ratio of reduced and oxidized biopterin, is important for determining the rates of NO production versus uncoupled superoxide formation from eNOS.[60,70] Only the completely reduced (tetrahydro) form of biopterin supports NOS coupling of NADPH oxidation to NO synthesis. Partially oxidized analogues of BH4 enhance rates of superoxide formation from purified eNOS in the presence of saturating L-arginine concentration.[58] Therefore, oxidative stress causes "uncoupling" of eNOS not only by decreasing BH_4 levels but also by increasing the ratio of BH4/7,8-dihydrobiopterin. Then, generation of superoxide and peroxinitrite from dysfunctional (uncoupled) eNOS induces a further reduction of BH4 availability.[54]

The mechanism of the improvement of endothelial dysfunction by vitamin C includes its effects on BH4.[71,72,73] Vitamin C not only scavenges superoxide but also enhances NO synthase activity. Vitamin C increases the Kmax of NOS enzyme without any effects on L-arginine. It is postulated that, by its reductase capacity, vitamin C chemically stabilizes BH4, but a recent study of Kuzkaya et al showed that vitamin C reduces the intermediate product of the reaction between peroxynitrite and BH4, BH3, back to BH4.[74] Saturated ascorbic acid levels in endothelial cells are necessary to protect BH4 from oxidation to provide optimal condition for cellular NO synthesis.

eNOS and Atherogenesis

As described, it seems to be established now that in hyperlipidemia and atherosclerosis, eNOS is dysfunctional and produces superoxide, which is implicated in endothelial dysfunction and impaired EDR. However, only limited information is available on how eNOS dysfunction affects atherogenesis. A substantial body of evidence in vitro suggests that eNOS-derived NO acts as anti-atherogenic molecule.[75–78] NO from eNOS inhibits leukocyte–endothelial adhesion, vascular smooth muscle migration and proliferation, and platelet aggregation, all of which are important steps in atherogenesis. Although the exact mechanisms are still not well defined and although there is still some controversy, chronic treatment with L-arginine, a substrate for NOS, inhibits atherosclerotic lesion formation in animal models of atherosclerosis, such as diet-induced atherosclerosis models of rabbits and LDL-receptor knockout mice.[79,80] On the contrary, NOS inhibitors like L-NAME significantly accelerate atherosclerotic lesion development, suggesting that inhibition of endogenous NO synthesis facilitates the progression of atherosclerosis.[81,82] Although little information is available for NOS gene transfer in atherosclerotic lesion formation, local adenovirus-mediated nNOS gene transfer to atherosclerotic carotid arteries rapidly reduces adhesion molecule expression and inflammatory cell infiltration in cholesterol-fed rabbits, indicating an anti-atherogenic role of endogenous NO in vivo.[83]

eNOS Gene Engineered Mice as a Tool to Study the Role of eNOS in Atherogenesis

Recently, eNOS gene-engineered mice have been used to clarify more directly the role of eNOS/NO system on atherogenesis. Knowles et al first demonstrated that a genetic lack of eNOS resulted in enhanced atherosclerosis in association with hypertension in apo E/eNOS double-knockout mice, which were produced by crossing apo E-KO mice with eNOS knockout (eNOS-KO) mice.[84] Based on the positive correlation between blood pressure and the size of atherosclerotic lesions in aortas, they suggested that an elevation of blood pressure was responsible for the increases in the lesion size in these mice. More recently, their group reported that the hypertensive and atherogenic effects of eNOS deficiency in apoE-KO mice depended on the presence of endogenous sex hormones.[85] By use of gonadectomized apo E/eNOS double-knockout mice, they suggested that in the absence of sex hormones, eNOS had little effect on blood pressure and atherogenesis, although which hormones were responsible for these effects were not identified. Kuhlencordt et al also reported that eNOS deficiency promoted atherosclerosis in apo E/eNOS double-knockout mice.[86] Fed with a "Western-type" diet, apo E/eNOS double-knockout mice showed significant increases in aortic lesion area, which were associated with peripheral coronary atherosclerosis and aortic aneurysm formation. Later, they showed that these changes were not inhibited by hydralazine treatment, which reduced blood pressure to the levels comparable to those of apoE-KO mice and concluded that hypertension did not account for the accelerated atherosclerosis and aortic aneurysm formation.[87] Therefore, although the participation of elevated blood pressure and sex hormones remains to be further clarified, these reports indicated that the absence of endogenous eNOS-derived NO caused by the lack of eNOS gene accelerates atherosclerosis.

In contrast, recently Shi et al reported the paradoxical reduction of atherosclerotic lesion size in high-cholesterol diet-induced atherosclerosis in eNOS-KO mice compared with wild-type mice.[88] They fed mice a "high-cholesterol diet" for 12 weeks and then examined the lesion size in the aortic sinus. They found that eNOS-KO mice had much smaller aortic sinus lesions than did wild-type mice. L-NAME, the NOS inhibitor, reduced LDL oxidation by endothelial cells from wild-type mice but not from eNOS-KO mice. Based on these findings, they speculated that eNOS may contribute to the oxidation of LDL under the circumstance of hypercholesterolemia, and that the absence of eNOS-mediated LDL oxidation may lead to the reduction of atherosclerotic lesion formation in eNOS-KO mice. They did not refer to the mechanisms of eNOS-mediated LDL oxidation, but it is very likely that superoxide from the dysfunctional eNOS was involved in the mechanisms. This study raised the possibility that eNOS may act to accelerate atherogenesis under certain conditions such as hypercholesterolemia.

We have examined the effects of eNOS overexpression on atherosclerotic lesion formation with the use of transgenic (eNOS-Tg) mice that overexpress eNOS mainly in the endothelium.[89,90] We crossed eNOS-Tg mice with apo E-KO mice and fed them a "high-cholesterol diet." Unexpectedly, the atherosclerotic lesion areas were significantly larger in eNOS-overexpressing apo E-KO (apo E-KO/eNOS-Tg) mice compared with control apo E-KO mice.[63] In apoE-KO/eNOS-Tg mice, we found the presence of eNOS dysfunction, demonstrated by lower NO production relative to eNOS protein levels and enhanced superoxide production in the endothelium. We also found decreased vascular BH4 levels and increased 7,8-dihydrobiopterin levels in apo E-KO/eNOS-Tg mice. Therefore, chronic overexpression of eNOS does not inhibit, but rather accelerates atherosclerosis under hypercholesterolemia. In contrast, van Haperen et al also crossbred apo E-KO mice with another line of eNOS transgenic mice that they created and reported that atherosclerotic lesion size was reduced by eNOS overexpression.[91] Regarding the mechanisms, they cited the reductions of blood pressure and plasma cholesterol levels. In their study, eNOS overexpression was associated with 20- to 25-mm Hg reduction in mean blood pressure and a ≈15% decrease in plasma cholesterol levels. Although the difference in promoter by which eNOS was targeted to the endothelium is possibly involved, the discrepancy between their study and ours can be explained at least partly by a difference in the balance between NO and superoxide production from the endothelium. The increase of plasma cholesterol levels achieved by the "Western-type" diet that they used was much modest compared with that we achieved by feeding a "high-cholesterol" diet. Therefore, it is speculated that oxidative stress in the hypercholesterolemic mice of van Haparen et al was not increased as much as that in our model, although they did not describe oxidative stress and eNOS function in their model.

As mentioned, increasing evidence demonstrates the presence of eNOS dysfunction in hyperlipidemia and atherosclerosis. It is conceivable that dysfunctional eNOS may promote atherogenesis under certain pathological conditions that alter the balance between eNOS protein levels and tissue pteridine metabolism. Under pathological conditions with severe hyperlipidemia, there exists an increase in oxidative stress, which determines the extent of eNOS uncoupling and the resultant generation of superoxide from eNOS. In contrast to NO, superoxide is a pro-atherogenic molecule, and antioxidants have been demonstrated to inhibit atherosclerotic lesion formation.[92] The marked increase in superoxide in association with decreased NO production would promote atherogenesis. However, it is totally unclear whether acceleration of atherogenesis by dysfunctional eNOS occurs only under a specific condition with severe hypercholesterolemia or whether it may take place under other pathological conditions with elevated oxidative stress. The role of eNOS dysfunction on atherogenesis needs further studies (Table).

Therapeutic Implication

It is important to define a therapeutic intervention for atherosclerosis from the standpoint of dysfunctional eNOS. Although the role of BH4 in the regulation of eNOS function is still not well understood, supplementation with exogenous BH4 is effective for the treatment of endothelial dysfunction. We found that supplementation with BH4 inhibits atherosclerotic lesion formation in apo E-KO mice.[63] Although the detailed mechanisms are unclear, it is conceivable that in

Atherosclerotic Lesion Formation in eNOS Gene-Engineered Mice

Model of Atherosclerosis	Lesion Size	Reference
eNOS-KO Mice cross-breeding with apo E-KO mice (caused by hypertension or sex hormones?)	Augmented	84, 85
eNOS-KO Mice cross-breeding with apo E-KO mice (unrelated to hypertension)	Augmented	86, 87
eNOS-KO Mice, diet-induced atherosclerosis	Reduced	88
eNOS-Tg Mice, cross-breeding with apo E-KO mice	Augmented	63
eNOS-Tg Mice, cross-breeding with apo E-KO mice	Reduced	91

addition to the simple removal of superoxide by its antioxidant effect, exogenous BH4 improved pteridine metabolism at the vessel wall and led to restore normal eNOS function. However, the effect of sepiapterin on atherosclerosis lesion formation has not been reported yet and it may not be effective. It is necessary to further clarify pteridine metabolism in the tissues, particularly in the vascular wall. GTPCH could be a rational target to augment endothelial BH4 and normalize eNOS activity in endothelial dysfunction. As for the strategy for augmenting GTPCH activity, GTPCH 1 gene transfer in vitro to human endothelial cells augments intracellular BH4 levels in association with an increase in enzymatic activity of eNOS to produce NO.[93] Recently, Alp et al generated transgenic mice overexpressing GTPCH I solely in the endothelium.[94] They reported that in the rat model of streptozotocin-induced diabetes, overexpression of GTPCH I augmented endothelial BH4 levels, improved the impaired vascular function, and decreased superoxide production from vessels. They suggested that a small increase in BH4 levels in the tissue was sufficient to maintain normal eNOS function. The beneficial effects of GTPCH I gene transfer was also

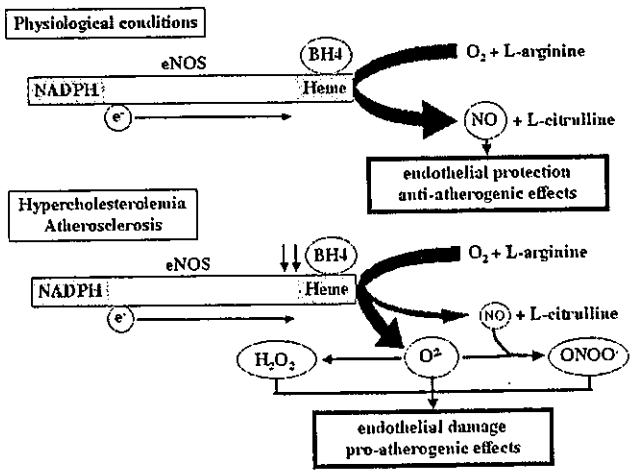

Figure 2. Hypothetical scheme illustrating the possibility of divergent roles of eNOS in atherogenesis. Under physiological conditions, tissue levels of BH4 are optimal for eNOS catalytic activity, and activation of eNOS generates NO and L-citrulline. NO generated by eNOS serves as an anti-atherogenic molecule. With hypercholesterolemia and atherosclerosis, when oxidative stress is increased, tissue levels of BH4 are reduced. In the presence of suboptimal levels of BH4, activation of eNOS leads to "uncoupling of NOS" with subsequent generation of superoxide rather than NO. Superoxide and, subsequently, peroxinitrite and hydrogen peroxide serve to damage endothelial cells and thus may promote atherosclerosis.

confirmed by a very recent study of Zheng et al, who reported that ex vivo gene transfer of human GTPCH I to the aortic segments from DOCA-salt hypertensive rats reversed BH4 deficiency in the vascular tissue and improved EDR.[95]

The anti-atherogenic property of drugs may also be evaluated from the standpoint of their effects on GTPCH. Statins are shown to increase eNOS protein levels in endothelial cells. Hattori et al demonstrated that statins increased GTPCH I mRNA in vascular endothelial cells and led to an elevation of intracellular BH4 levels.[96] These effects may be partly responsible for the anti-atherogenic action of statins.

However, simply augmenting NOS protein levels under pathological conditions such as hyperlipidemia may not increase NO but instead augment superoxide production, resulting in detrimental rather than beneficial effects. Therefore, a strategy directed at increasing NOS protein levels in association with maintaining its enzymatic activity is needed.[97,98] (Table 1, Figure 2)

Summary

It is now being widely recognized that eNOS becomes dysfunctional and produces superoxide rather than NO in hyperlipidemia and atherosclerosis. Dysfunctional eNOS is closely implicated in the endothelial dysfunction represented by impaired EDR in atherosclerotic vessels. It seems to be widely accepted that eNOS with normal function inhibits atherogenesis by producing NO. However, although further studies are needed, recent reports on eNOS gene-engineered mice raised the possibility that dysfunctional eNOS may serve to promote atherosclerotic lesion formation under severe hypercholesterolemia (Figure 2). For the development of eNOS dysfunction, an abnormality in BH4 metabolism in vascular tissue seems to be fundamental. However, little is known about BH4 metabolism in vascular tissue, particularly in diseased states including atherosclerosis. We need an improved understanding of tissue BH4 metabolisms in atherosclerotic vessels in relation to conditions in which eNOS dysfunction develops. It would be intriguing to know whether dysfunctional eNOS participates in the pathogenesis of vascular disorders other than atherosclerosis.

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