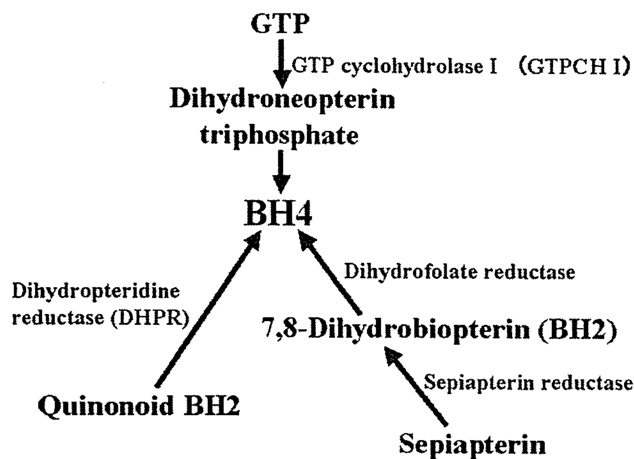

Figure 1. Scheme of BH4 biosynthesis.

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 $\approx 15\%$ 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 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]

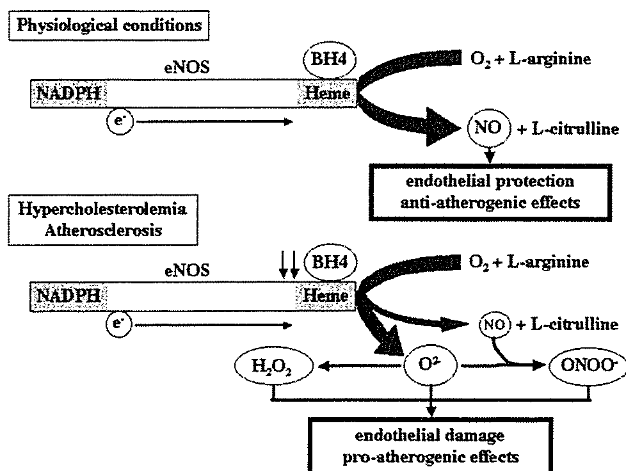

Figure 2. Hypothetical scheme illustrating the possibility of divergent roles of eNOS in atherogenesis. Under physiological conditions, tissue levels of BH4 are optimal for eNOS catalytic activity, and activation of eNOS generates NO and L-citrulline. NO generated by eNOS serves as an anti-atherogenic 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.

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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一酸化窒素の抗動脈硬化作用を探る

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生体において，一酸化窒素（NO）は 3 種の NO 合成酵素（NOS）により産生され，そのなかで動脈硬化をはじめとした血管病と最も関係するのは，内皮型 NOS（eNOS）により産生される NO である．NO はその多彩な作用により，抗動脈硬化分子として作用すると *in vitro* の研究結果より考えられてきたが，近年，種々の NOS 遺伝子操作マウスの研究からも明らかになってきた．また，ヒトにおいても NO が抗動脈硬化にはたらくことは，eNOS 由来 NO によりもたらされる内皮依存性血管弛緩・拡張反応（EDR）の減弱の程度が，その後の心血管イベントの予知因子となることからも推察される．しかしながら高脂血症・動脈硬化においては，NOS の補酵素テトラヒドロビオプテリン（BH_4）の血管壁での含量の低下が生じるため，eNOS が効率的に NO を産生せず，スーパーオキシド（$O_2^{\cdot -}$）を産生する．その結果，NO の作用が減弱する．eNOS と BH_4をともに増加させるような治療法が，動脈硬化の進展防止には有効と考えられる．

KEY WORDS

一酸化窒素　動脈硬化　一酸化窒素合成酵素　テトラヒドロビオプテリン（BH_4）
遺伝子操作マウス

はじめに

血管内皮は多様な分子を産生・分泌し血管機能を調整しており，それ自身が重要な一種の臓器であると考えられる．内皮の作用には，血管トーヌスの調節，血液の凝固線溶の制御，平滑筋細胞の増殖の制御などさまざまなものがあるが，全体として血管内皮は血管構築の維持，血管傷害からの防御にはたらいているものである．そして内皮機能がさまざまな疾患で障害され，それが各疾患の病態形成に深く関与していることが明らかになってきた．

内皮が産生・分泌する物質の代表が一酸化窒素（NO）である．NO は多彩な機能を有すガス状分子であるが，心血管領域において NO 研究が急速に広がったのは，内皮由来血管弛緩因子（endothelium-derived relaxing factor：EDRF）の本体が NO であり，内皮依存性血管弛緩・拡張反応（endothelium-dependent relaxation：EDR）が

NO によってもたらされることが解明されたことによる．高脂血症・動脈硬化との関連において，はじめに NO の役割が注目されたのは，これらの病態における EDR の減弱であり，その後，NO の多彩な生物作用が明らかになるにしたがい，NO は EDRF として作用する以外に，動脈硬化の発症・進展にも深く関与していると考えられるようになってきた．

本稿では動脈硬化における NO の役割，関与について，まず EDRF としての NO の作用，さらに重要な動脈硬化修飾分子としての NO の作用を述べる．

多彩な機能分子 NO は抗動脈硬化分子である

NO は L-アルギニンと酸素を基質として，NO 合成酵素（NOS）により産生される．NOS には内皮型 NOS（eNOS），誘導型 NOS（iNOS），脳神経型 NOS（nNOS）の 3 種のアイソフォームがあり，血管においては内皮細胞に eNOS が，おもに外膜の神経末端に nNOS が存在し，血管への傷害や炎症が生じた場合には浸潤炎症細胞や平滑筋細胞に iNOS が誘導される．なかでも，内皮細胞に存在する eNOS は，血流のずり応力や，血中のブラジキニン，血管内皮細胞増殖因子（vascular endothelial growth factor：VEGF），インスリン，アセチルコリンなどにより，常に活性化され NO を恒常的に産生しており，血管トーヌスの調整，血管構築の維持に重要な役割を果たしている[1]．

NO には EDRF として血管の弛緩・拡張を調整する作用以外に，**表❶**に示すような多彩な機能があり，とくに内皮細胞-白血球接着の抑制，平滑筋細胞の増殖・遊走の抑制，血小板凝集の抑制作用は，それぞれ動脈硬化の発症・進展の重要な過程にはたらいている[2)3)]．なかでも内皮細胞への単球・白血球の接着は，その後これらの細胞が内皮下に侵入し粥腫を形成するうえでの必須なステップであるが，NO は内皮細胞ならびに白血球における接着分子の発現を抑制し，この接着を抑制する．これは高脂血症家兎において，NOS 遺伝子の血管壁への導入が内皮での接着分子発現ならびに血管壁への白血球浸潤を抑制したことからも明らかである[4)]．

表❶　NO の多彩な作用のうち血管病（とくに動脈硬化）と関係するもの

1．内皮由来血管弛緩因子（EDRF）として血管トーヌスの調整
2．内皮細胞-白血球接着の抑制
3．平滑筋細胞の増殖・遊走の抑制
4．種々の遺伝子発現の調節
5．血小板の凝集抑制
6．LDL の酸化抑制
7．アポトーシスへの作用（内皮由来の比較的少量の NO は抑制，大量の NO では誘導）

（筆者作成）

また，種々の血管リモデリングの発症機序は動脈硬化の発症機序と類似しており，とくに新生内膜形成においては平滑筋細胞の増殖・遊走が中心的役割を果たす．この新生内膜形成を，種々の NOS 遺伝子の血管壁への導入が抑制すること，さらには後述の eNOS 遺伝子操作マウスにおいて，eNOS 遺伝子欠損，ならびに過剰発現が新生内膜をそれぞれ増強，抑制することも，生体において NO が抗動脈硬化分子として作用することを示唆するものである[5)]．また，動脈硬化の進展には LDL が酸化変性を受けることが必須と考えられるが，この LDL 酸化を NO が抑制することも考えられる[6)]．このような機能により，NO は抗動脈硬化分子としてはたらくと想定されている．

動脈硬化血管では eNOS/NO 系はどうなっているのか？

高脂血症・動脈硬化において EDR が減弱していることはよく知られている[7)]．この機序にはさまざまなことが考えられているが，主たるものは eNOS の酵素活性の低下，ならびに産生された NO がスーパーオキシド（O_2^-）により不活性化されることであり，その結果，血管壁からの NO 量が減少している[8)]．また最近の報告では，動脈硬化血管では平滑筋細胞での NO の標的分子である可溶性グアニル酸シクラーゼ（sGC）の活性が低下し，さらには sGC により産生された cyclic GMP（cGMP）が作用する cGMP 依存性キナーゼ（cGK）の発現も低下し

ていることが報告されている．すなわち，動脈硬化血管ではNOばかりでなく，eNOS/NO系の細胞内シグナルそのものも低下している可能性がある[9]．

一方，内皮におけるeNOS発現に関しては諸説があるが，動脈硬化の初期においては，EDRの減弱にもかかわらずeNOS発現は増加しており，動脈硬化病変が進行するとeNOS発現は減弱するとの考えが一般的であると考えられる[10]．これに対し，iNOSは動脈硬化の初期では血管壁での発現はほとんど認められない．そして動脈硬化病変が進展するにしたがい，主として粥腫に浸潤した炎症細胞・マクロファージを中心として発現する．また，iNOS発現部位と近接して，強い細胞傷害性を示すペルオキシナイトライト（$ONOO^-$）の産生産物が認められる．また，nNOSに関しての報告はあまり見当たらないが，やはり正常血管ではほとんど発現が認められず，進行した動脈硬化病変では，内皮や浸潤炎症細胞に発現が認められるとの報告がなされている[11]．

遺伝子操作マウスにより明らかになったNOの抗動脈硬化作用とは？

生体において，それぞれのNOSより産生されるNOがどのように動脈硬化の発症・進展とかかわっているかに関しては，それぞれのNOS遺伝子欠損マウスを用いた研究により解明が進んだ．まずeNOSに関しては，eNOS遺伝子欠損マウスとapoE遺伝子欠損マウスの交配実験の結果が少なくとも2施設より報告されており，eNOS遺伝子が欠損することにより動脈硬化が増悪すると報告されている[12][13]．これは，生体においてeNOS/NO系が動脈硬化の抑制にはたらいていることを意味する．しかしながら，eNOS遺伝子欠損による動脈硬化病変の悪化は，血管局所における抗動脈硬化分子としてのeNOS由来NOの作用の消失によるだけではなく，eNOS欠損による血圧上昇も関係している可能性がある．一方，高コレステロール食負荷のマウス動脈硬化モデルにおいては，eNOS遺伝子欠損により，動脈硬化病変が軽減するという，これらの論文と相反する結果を示す報告も存在する[14]．

血管壁におけるもう一つのNOS，iNOSに関しては，iNOS由来の大量のNOは炎症惹起性にはたらき，LDL酸化に関係していることが実験的に示されている．そしてiNOS遺伝子欠損マウスとapoE遺伝子欠損マウスの交配実験が少なくとも3施設から報告されている[15][16]．高コレステロール食の負荷条件により多少結果は異なるが，iNOSの非存在下では動脈硬化病変が減少し，また粥腫の組成も線維成分の多いより安定したものへと変化するとされている．以上より，動脈硬化の進展に伴い発現するiNOSにより産生されるNOは，病変をさらに進行させ，粥腫をより不安定なものへと変化させているものと推定される．これらに対し，nNOSに関してはnNOS遺伝子欠損マウスとapoE遺伝子欠損マウスの交配実験をおこなったところ，動脈硬化が進行するとの結果が学会抄録[17]として報告されているが詳細は不明である．

ヒトにおいてNOが動脈硬化を抑制するという証拠はあるのか？

ヒトにおいて，NOが動脈硬化の発症・進展と関係するかどうかを検証することはなかなか困難である．この原因として，ヒトにおける動脈硬化の進行が緩徐であることに加え，内因性NOと同様の作用を血管壁にもたらすような外因性NOを投与することの困難さがあげられる．硝酸薬はNOを放出することにより作用することはよく知られているが，硝酸薬によって動脈硬化を抑制できたという報告は動物実験においてもほとんど見当たらない．最近，Mullerら[18]はイソソルビドの大量投与（200 mg/kg/日）が高コレステロール食負荷の家兎動脈硬化モデルにおいて，血管からのO_2^-産生を低下させ，動脈硬化病変を軽減すると報告している．しかしながら，大量の外因性NOの投与はNO耐性を血管に生じさせることも考えられるため，この報告の意義は今後検証されなくてはならないと考える．

一方，ヒトにおいてNOが抗動脈硬化作用を有すということを示す間接的証拠はいくつかあげられる．この場

表❷ 内皮機能が将来の心血管イベントの予知因子になることを示した報告のまとめ

	患者数	対象	対象血管	観察期間	結果
冠動脈					
Schächinger ら (2000)	147	診断カテーテル	心外膜側冠動脈	82 カ月	Ach/FMD 反応は心血管イベントの独立した予知因子
Halcox ら (2002)	308	診断カテーテル	心外膜側冠動脈および微小冠動脈	46 カ月	Ach 反応は心血管イベントの独立した予知因子
Al Suwaidi ら (2000)	157	冠動脈疾患患者	微小冠動脈	28 カ月	Ach による拡張の減弱した患者で心血管イベント発症
末梢血管					
Heitzer ら (2001)	281	冠動脈疾患患者	前腕抵抗血管	54 カ月	Ach 反応は心血管イベントの独立した予知因子
Perticone ら (2001)	225	高血圧患者	前腕抵抗血管	31 カ月	Ach 反応は心血管イベントの独立した予知因子
Gokce ら (2000)	187	血管手術前患者	とう骨動脈	1 カ月	FMD 反応は術後の心血管イベントの独立した予知因子
Neunteufl ら (2000)	77	胸痛患者	とう骨動脈	60 カ月	FMD 反応は心血管イベントの独立した予知因子

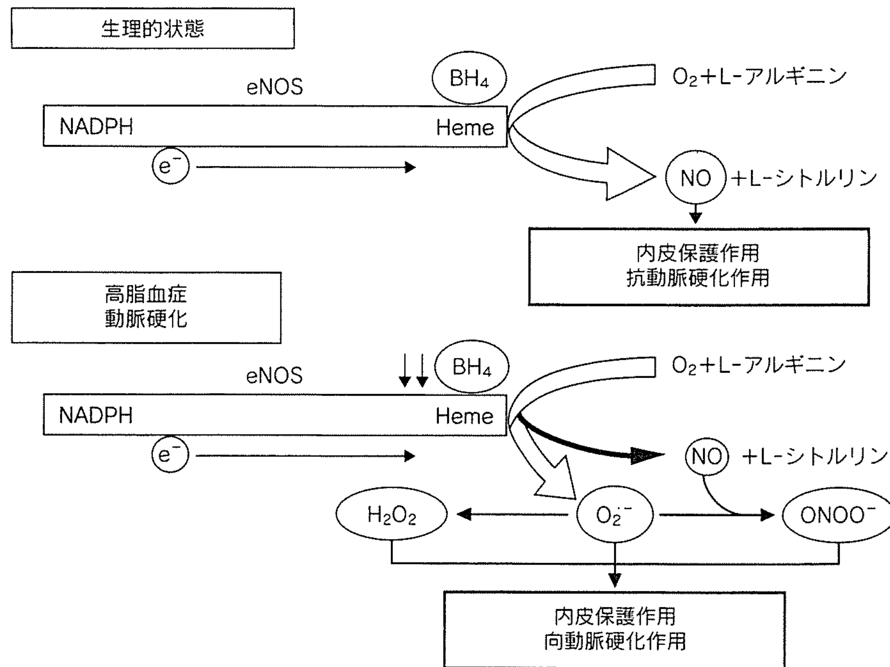
Ach：アセチルコリンの冠動脈あるいはとう骨動脈内への注入による血管拡張反応.
FMD：血流依存性の血管拡張反応.

(Landmesser U *et al*：*Ciculation* **109** (suppl 1)：S27-S33, 2004 より改変引用)

合，EDR が重要な役割を果たす．すでに述べたように，EDR の減弱は内皮機能障害，そして eNOS により産生される NO 作用の低下を示すものである．すでにスタチンやアンジオテンシン変換酵素（ACE）阻害薬あるいはアンジオテンシン受容体拮抗薬（ARB）による，血管内皮での eNOS 発現の増加に伴う EDR の改善，動脈硬化病変の進展抑制，さらには動脈硬化性疾患が主たる原因と考えられる心血管イベントの減少が報告されている．これらの薬剤は，内皮細胞への作用以外にも，血管に対し多彩な作用を有すため，これらの薬剤による動脈硬化病変の抑制効果が，すなわち eNOS/NO 系の増強効果を介したものであるとはいえないが，やはり内皮細胞由来 NO の抗動脈硬化作用を示唆するものである．

一方，この数年，血管内皮機能障害の程度が冠動脈疾患患者の予後に関係するとの報告がなされている（**表❷**）．2002 年に Halcox ら[19]は約 300 名の患者の平均 4 年間の追跡調査において，冠動脈内にアセチルコリンを投与した場合の血管反応と，観察期間における心血管イベント発症との関連を調べた．その結果，冠動脈抵抗血管での拡張反応の低下群，および冠動脈心外膜側の太い部位が拡張せずに収縮する群において，その後の心血管イベントの発症が多いことを報告した．この報告に先立って，Schächinger ら[20]は平均 7 年間，最長 10 年間の追跡調査により，初回解析時の冠動脈病変の有無にかかわらず，冠動脈心外膜側の血管の種々の刺激に対する拡張反応の程度が，その後の心血管イベント発症の予知因子となりうることを報告した．この場合興味深いことに，アセチルコリンや血流増加に対する拡張反応の低下，すなわち内皮依存性の血管拡張反応の低下のみならず，ニトログリセリンによる血管拡張反応，すなわち内皮に依存しない反応までもが，心血管イベント発症の予知因子になることが明らかにされた．これは前述の家兎の進行した動脈硬化血管では，内皮のみならず平滑筋レベルにおいても NO に対する拡張反応性が低下しているとの報告と合致するものであり，動脈硬化においては内皮同様血管平滑筋にも機能異常が生じていることを示唆する所見と考えられる．さらにその後，冠動脈ばかりでなく，前腕動脈での血流依存性の血管拡張反応（flow mediated dilatation：FMD）で判定した内皮機能が，心血管イベント発症に関係することもいくつかの施設から報告されてきている[21]（**表❷**）．

これらの報告は，心血管イベントの基盤に存在する動脈硬化病変の進展が，内皮機能に大きく影響を受けていることを意味すると解釈される．実際に，前述の Schächinger らの報告[20]においても，冠動脈造影において有意な狭窄病変を示さない冠動脈局所の経時的な観察において，



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

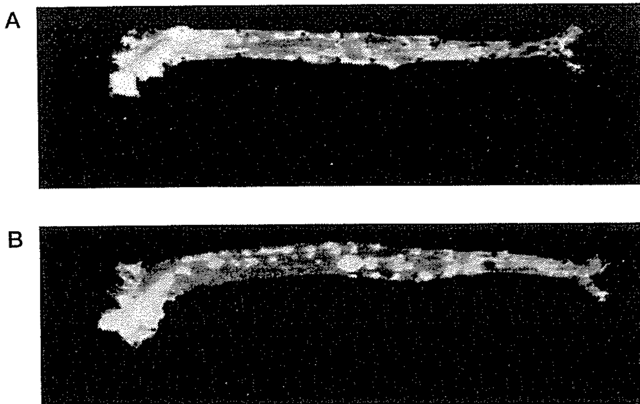
その後の追跡冠動脈造影でアセチルコリンにより血管が拡張せずに収縮した部位，すなわち内皮が傷害され EDR が減弱している部位に，器質的狭窄病変が出現したと述べられている．そして，内皮機能のうち最も重要なものが eNOS 由来の NO 産生であることより，これら一連の臨床研究の結果は，eNOS 由来 NO が，動脈硬化の防止・抑制にはたらいていることを示唆する所見と理解される．

動脈硬化に対して eNOS は抑制作用をもたらすだけなのか？

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

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

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



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

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

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

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

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

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

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

おわりに

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

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Angiotensin II type 1 receptor blocker telmisartan suppresses superoxide production and reduces atherosclerotic lesion formation in apolipoprotein E-deficient mice

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Abstract

Angiotensin II is involved in the process of atherosclerosis and stimulates superoxide production from cardiovascular cells. We examined the effect of telmisartan, an angiotensin II type 1 receptor blocker, on atherosclerosis. We chronically treated apolipoprotein E-deficient mice with two different doses of telmisartan dissolved in drinking water (0.3 and 3 mg/kg) starting from 4 weeks of age for 12 weeks. Lipid contents were not different in both telmisartan-treated groups compared with control group. Systolic blood pressure was significantly reduced with 3 mg/kg, but unchanged with 0.3 mg/kg. The total atherosclerotic lesion size at the aortic sinus was reduced with 0.3 mg/kg compared with control, and additional reduction was proved with 3 mg/kg. The fibrotic change was not different among three groups, but MOMA-2-, malondialdehyde-, 4-hydroxy-2-nonenal-immunostained areas were reduced by telmisartan. As the mechanism, we revealed that both doses of telmisartan markedly reduced superoxide production from in situ vessels assessed by lucigenin-enhanced chemiluminescence and dihydroethidium staining. And NAD(P)H dependent oxidase activity in vessels was reduced by telmisartan. Further, 8-*iso*-prostaglandin F2α level, a systemic oxidative stress marker, obtained from urine and plasma samples were significantly reduced by telmisartan. Telmisartan reduced atherosclerosis in apolipoprotein E-deficient mice at least partly via the suppression of oxidative stress.
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Keywords: Angiotensin II type 1 receptor blocker; Atherosclerosis; Oxidative stress; Apolipoprotein E-deficient mouse

1. Introduction

The renin-angiotensin system (RAS) plays important roles in the regulation of not only blood pressure but also vascular structure. The main component of RAS is angiotensin II (Ang II), which is a potent vasoconstrictor and elevates blood pressure. Ang II generates aldosterone and activates sympathetic nervous system, leading to blood pressure elevation.

Besides its effect on blood pressure, a number of evidence revealed that Ang II is involved in atherogenesis. In animal models, chronic infusion of Ang II promotes atherosclerotic lesion formation [1]. It is shown that Ang II promotes atherogenesis via direct effects on vascular beds independent of hypertensive effects. Among them, the effect as the inducer of oxidative stress is recently attracting attention. In atherosclerosis, there is augmented production of reactive oxygen species (ROS) from various cell types including endothelial cells, vascular smooth muscle cells and monocytes/macrophages, and Ang II plays a pivotal role in their production [2–4]. There are increased expressions of angiotensin converting enzyme (ACE) and Ang II type 1 receptor in atherosclerotic arteries, indicating the presence of augmented local RAS activation [5,6]. Ang II increases superoxide production from vessel wall by activating NAD(P)H oxidase [7]. ROS are closely implicated

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in atherogenesis, by damaging and activating the endothelium, oxidizing low-density lipoprotein (LDL) cholesterol, and promoting proliferation of vascular smooth muscle cells. They also induce various genes such as those of adhesion molecules and chemokines, which play important roles in the initiation and progression of atherosclerotic lesion formation [8,9].

ACE inhibitors (ACE-I) and Ang II type 1 receptor blockers (ARB) are widely used for treatment of hypertension to prevent organ damages. These drugs have been already reported to prevent atherosclerosis in several studies using animal models [10–13]. Losartan reduced atherosclerotic lesion formation without changing blood pressure in cynomologous monkeys [10] and apolipoprotein E-deficient (apoE-KO) mice [11]. Olmesartan was reported to reduce atherosclerosis in association with suppressions of serum macrophage-colony stimulating factor, transforming growth factor-beta 1 and intracellular adhesion molecule-1 in monkeys fed a high cholesterol chow [13].

There has been little information whether ARB can show anti-atherogenic effects by the mechanisms related to suppression of oxidative stress. In the present study, we investigated the effects of telmisartan, an ARB, on atherosclerotic lesion formation in apoE-KO mice. Particularly, we examined whether the effects of telmisartan on atherogenesis were independent of its effect on blood pressure and associated with changes in oxidative stress.

2. Materials and methods

2.1. Materials and animal preparation

Telmisartan was obtained from Boehringer Ingelheim Inc. (Germany). All other commercial drugs used in this study were purchased from Sigma Chemical Co. (MO). ApoE-KO mice on a C57BL/6 genetic background at 4 weeks of age were assigned to control group and two telmisartan-treatment groups given different dosages. Drug treatment consisted of 0.3 and 3 mg/kg body weight per day of telmisartan dissolved in drinking water. Mice were fed a standard chow and supplemented with telmisartan for next 12 weeks and sacrificed at 16 weeks of age. Animals were provided the chow and water ad libitium and maintained on a 12 h light/dark cycle. All animal experiments were conducted according to the guidelines for animal experiments at Kobe University Graduate School of Medicine.

2.2. Plasma analysis

After overnight fasting, blood was collected by the cardiac puncture into heparin-coated tubes under anesthetic condition using pentobarbital sodium (80 mg/kg intraperitoneal injection). Plasma was obtained through centrifugation of the blood for 10 min at $5500 \times g$ at 4 °C and stored at −80 °C until each assay. Concentrations of plasma total cholesterol and triglyceride were determined by use of an automated clinical chemistry analyzer. High-density lipoprotein cholesterol levels were quantified by enzymatic reaction using a commercially available kit (Wako, Japan). Glucose levels were determined by glucometer (Sanwa Kagaku, Japan) and insulin levels were determined with a commercially available kit (LINCO Research Inc., MO).

2.3. Hemodynamic analysis

Heart rate and systolic blood pressure of apoE-KO mice were measured at 16 weeks of age using the tail-cuff method without heating. The mouse tail was placed into a device with a rubber cuff and a photoelectric sensor, and heart rate and systolic blood pressure were measured using MK-2000 (Muromachi Kikai, Japan). All measurements were repeated six times for each mouse.

2.4. Atherosclerotic lesion assessment at the aortic sinus

After 12 weeks of telmisartan treatment, both gender mice (16 weeks of age) were anesthetized as above and the aorta was perfused with normal saline containing 10 U/ml heparin. Then the aorta sample was dissected from the middle of the left ventricle to the aortic arch, and fixed with 4% paraformaldehyde for overnight. The sample was cut in the ascending aorta, and the proximal sample containing the aortic sinus was embedded in OCT compounds (Tissue-Tek, CA). Five consecutive sections (10 μm thickness), spanning 550 μm of the aortic sinus, were collected from each mouse and stained with Sudan III and Masson's trichrome. For quantitative analysis of atherosclerosis, the average lesion area of five separate sections from each mouse was obtained with the use of the Image J (National Institutes of Health, MD) according to the method described by Paigen et al. [14].

2.5. Immunohistochemistry

Immunohistochemical staining with MOMA-2 (BMA Biomedicals AG, Switzerland; 1:500 dilution), malondialdehyde (MDA) (Alpha Diagnostic International Inc., TX; 1:100 dilution) and 4-hydroxy-2-nonenal (HNE) (Alpha Diagnostic International Inc., TX; 1:100 dilution) of atherosclerotic lesions at the aortic sinus was performed by the labeled streptaviadin biotin method as previously reported [15]. Quantitative analysis of MOMA-2-immunostaining was evaluated as a ratio of the positive-stained area to total plaque area in the atherosclerotic lesion at the aortic sinus.

2.6. Measurement of superoxide production from aortas

After euthanization of mice, the aorta was cut out from the aortic arch to the bifurcation of iliac arteries and the tissues around the vessel were cleaned. Then the aorta were cut into four pieces (approximately 5 mm length per each pieces) and

these aortic rings were incubated with the Cu–Zn superoxide dismutase inhibitor for 30 min at 37 °C, and vascular superoxide production levels were measured by chemiluminescence (CL) with 10 μM lucigenin (bis-*N*-methylacridinium nitrate). The final volume of lucigenin solution was 1 mL. The light reaction between superoxide and lucigenin was detected with a BLR-201 CL reader (ALOKA, Japan) and photon emission was continuously recorded for 15 min. The CL signal was expressed as the average count per minute (C.P.M.) for 15 min periods and the counts were corrected by vessel dry weights.

2.7. Measurement of NAD(P)H dependent oxidase activity of aorta homogenates

The aorta was cut out from each mouse as described above, and the aortic segments (almost 2 cm length) were placed in a chilled modified 50 mM HEPES/PSS buffer and homogenized on ice with a motor-driven tissue homogenizerfor 1 min in 200 μL homogenate buffer, which contained 0.01 mM EDTA. The homogenates were centrifuged at 1000 × *g* for 10 min. The pellet was discarded and the supernatant was stored on ice until use. Protein concentration of aorta homogenate was measured by the method of Bradford [16]. The assay solution contained 50 mM HEPES/PSS (pH 7.4), 1 mM EDTA, 6.5 mM $MgCl_2$, 83 mM sucrose, and 250 μM lucigenin as the electron acceptor and 100 μM NADH or 100 μM NADPH as the electron donor [17]. After pre-incubation at 37 °C for 20 min, the reaction was started by adding 20 μL of aorta homogenates. All CL data were evaluated after subtracting the CL counts obtained in the absence of homogenates. Each count was corrected by protein levels of aorta homogenate.

In some experiments, we examined the effects of 100 μM diphenylene iodonium (DPI), an inhibitor of all flavoenzymes, and 500 μM apocynin, an inhibitor of NAD(P)H oxidase, on superoxide production after stimulation of homogenates with NAD(P)H. The aorta homogenates were pre-incubated with each agent for 15 min before CL measurement.

2.8. In situ detection of superoxide production in aortas and endothelial cells

To evaluate in situ superoxide production from vessels, unfixed frozen cross sections of aortas were stained with dihydroethidium (DHE; Molecular Probe, OR) according to the previously validated method [18]. In the presence of superoxide, DHE is converted to the fluorescent molecule ethidium, which can then label nuclei by intercalating with DNA. Briefly, the unfixed frozen tissues were cut into 10 μm thick sections, and incubated with 10 μM DHE at 37 °C for 30 min in a light-protected humidified chamber. The images were obtained with a laser scanning confocal microscope (Carl ZEISS, Germany). Superoxide production was demonstrated by red fluorescence labeling.

For quantification of ethidium fluorescence from endothelial cells, fluorescence (intensity × area) was measured only on the luminal side of the internal elastic lamina using the Image J in high-power (100×) images [19]. For each vessel, total fluorescence was calculated from three separate high-power fields taken in each section of the vessel to produce $n = 1$.

2.9. Measurement of 8-iso-prostaglandin F2α and serum amyloid A levels

Urine samples were collected from mice at the age of 12–16 weeks, and stored at −80 °C after addition of butyrated hydroxytoluene (BHT) at a final concentration of 0.01%. After purification using C18 reverse phase extraction column (Waters Corporation, MA), urine 8-*iso*-prostaglandin (PG) F2α levels were measured with EIA kits (Assay Designs Inc., MI) according to the manufacturer's instructions, and data were corrected by urine creatinine levels. Plasma samples were collected as above and stored at −80 °C after addition of BHT at a final concentration of 0.01%. We measured direct 8-*iso*-PGF2α levels from plasma samples with EIA kits (Assay Designs Inc., MI) according to the manufacturer's instructions.

We collected plasma from each mouse as shown in Section 2 and measured serum amyloid A (SAA) levels with mouse SAA ELISA kit (BioSource International Inc., CA) according to the manufacturer's instructions.

2.10. Statistical analysis

Data were expressed as mean ± S.E.M. One-way ANOVA was used to compare the differences among three groups with Fisher's PLSD test for post hoc analysis. Values of $P < 0.05$ were considered statistically significant.

Table 1
Effect of telmisartan on body weight, lipid contents, glucose and insulin levels, and vital signs

	Control	0.3 mg/kg	3 mg/kg
Female body weight (g)	21.8 ± 0.5	21.2 ± 0.3	21.2 ± 0.5
Male body weight (g)	26.9 ± 0.6	27.0 ± 0.4	26.7 ± 1.5
Total cholesterol (mg/dl)	478.6 ± 28.8	463.4 ± 19.1	464.9 ± 22.8
Triglyceride (mg/dl)	53.5 ± 6.8	50.0 ± 9.7	57.8 ± 8.1
HDL cholesterol (mg/dl)	9.4 ± 0.8	9.3 ± 0.9	9.0 ± 0.7
Glucose (mg/dl)	108.4 ± 8.2	116.1 ± 7.1	112.5 ± 5.5
Insulin (ng/ml)	0.24 ± 0.12	0.18 ± 0.06	0.24 ± 0.05
Heart rate (min^{-1})	543.9 ± 50.8	536.3 ± 89.7	540.5 ± 76.7
Systolic BP (mmHg)	107.4 ± 1.7	106.9 ± 2.1	90.1 ± 1.8*

Mice were fed a standard chow for 16 weeks and body weight of each mouse was measured. Mice were fasted for at least 12 h and bled, and plasma total cholesterol, triglyceride, high-density lipoprotein cholesterol, glucose and insulin levels were determined as described in Section 2 ($n = 8$ per group). Heart rate and systolic blood pressure were measured with the use of the tail-cuff method ($n = 15$ per group). Results were expressed as mean ± S.E.M.; BP, blood pressure.

* $P < 0.0001$ vs. control.

3. Results

3.1. The effects of telmisartan on blood pressure and plasma lipid levels

Body weight was not significantly different among three groups of each gender (Table 1). Neither plasma total cholesterol, triglyceride, nor high-density lipoprotein cholesterol levels were affected by the treatment with telmisartan. And neither plasma glucose, nor insulin levels were affected by the treatment with telmisartan. Although heart rate was not affected, 3 mg/kg telmisartan significantly reduced systolic blood pressure compared with that of control group (Table 1). In contrast, 0.3 mg/kg telmisartan did not change systolic blood pressure.

3.2. Atherosclerotic lesion formation at the aortic sinus

After feeding a standard chow for 16 weeks, the atherosclerotic lesion formation was assessed at the aortic sinus. Representative photographs of each mouse were shown in Fig. 1A. In quantitative analysis of histological examination with Sudan III staining, the atherosclerotic lesion formation of both 0.3 and 3 mg/kg groups were markedly reduced compared with control group in both gender (Fig. 1B). About plaque contents, scattered small fibrotic area was distributed in the plaque lesions of both control and telmisartan treatment groups when evaluated by Masson's trichrome staining (Fig. 2). MOMA-2-immunostained area was significantly reduced with telmisartan, but a ratio of positive-stained

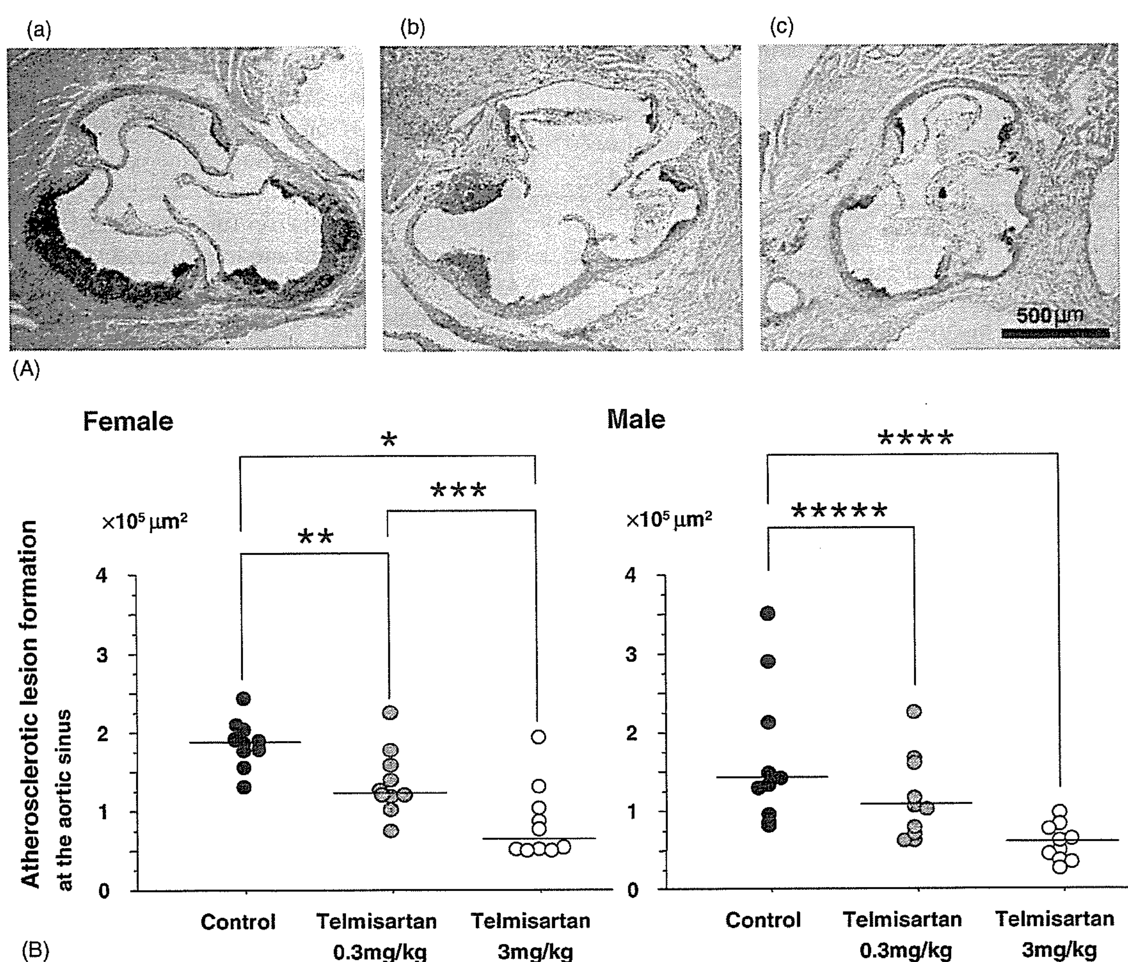

Fig. 1. (A) Representative photographs of atherosclerotic lesion formation at the aortic sinus of each mouse. Panel (a) through (c) are representative photographs of the atherosclerotic lesion formation at the aortic sinus of mice fed a standard chow in control group (a), that treated with 0.3 mg/kg telmisartan (b) and that treated with 3 mg/kg telmisartan (c), respectively. Sections were taken at the same level of aortic sinus and stained with Sudan III staining as described in Section 2. Original magnifications were 40×. A black bar on photomicrograph represents 500 μm. (B) Quantitative analysis of atherosclerotic lesion formation at the aortic sinus in both gender mice. The average lesion area of five sections at the aortic sinus from each mouse was quantified morphometrically as described in Section 2. Each symbol represents the average lesion area in each mouse, with the mean per group indicated by a horizontal line. After 12 weeks telmisartan treatment, the atherosclerotic lesion formation was significantly reduced in both 0.3 and 3 mg/kg telmisartan groups compared with control ($n = 10$ per group). $^{*}P < 0.0001$ vs. control; $^{**}P < 0.01$ vs. control; $^{***}P < 0.01$ vs. telmisartan 0.3 mg/kg; $^{****}P < 0.001$ vs. control; $^{*****}P < 0.05$ vs. control.

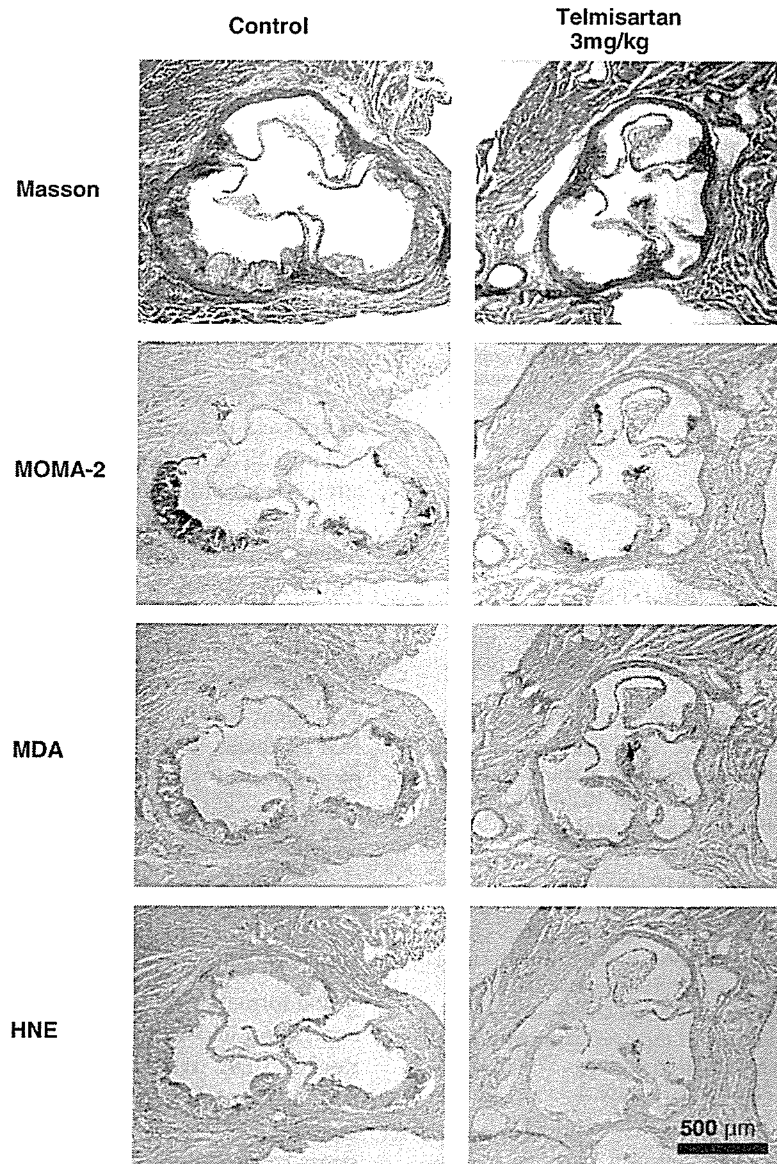

Fig. 2. Effect of telmisartan on plaque contents and the distribution of oxidative stress in atherosclerotic lesion at the aortic sinus. Panels are representative photographs of Masson trichrome staining and immunostained with MOMA-2, MDA and HNE in the atherosclerotic lesions from control group and 3 mg/kg telmisartan group (original magnification were 40×). Scattered fibrosis was partly distributed in the plaque lesions of both groups. On the whole, telmisartan reduced MOMA-2-, MDA- and HNE-immunostained areas compared with control group. A black bar on photomicrograph represents 500 μm.

area to total plaque area was not significantly different among three groups (data not shown). MDA-, HNE-immunostained areas were also reduced with telmisartan (Fig. 2).

3.3. Superoxide production from aortas

To investigate the effect of telmisartan on superoxide production in the aortic vessel wall, we measured superoxide production using the lucigenin-enhanced CL. By treatment with 0.3 and 3 mg/kg telmisartan, superoxide production was significantly decreased compared with control group (Fig. 3A).

3.4. NAD(P)H dependent oxidase activity of aorta homogenates

NAD(P)H dependent oxidase activity in aorta homogenates stimulated with 100 μM NADH or 100 μM NADPH was measured by use of the lucigenin-enhanced CL. Telmisartan significantly decreased NAD(P)H dependent oxidase activity by more than 60% in both 3 and 0.3 mg/kg groups compared with control group (Fig. 3B). Furthermore, aorta homogenates were incubated with either 100 μM DPI or 500 μM apocynin for 15 min to abolish the increment of NAD(P)H dependent oxidase activity. The addition of NAD(P)H oxidase inhibitors significantly reduced

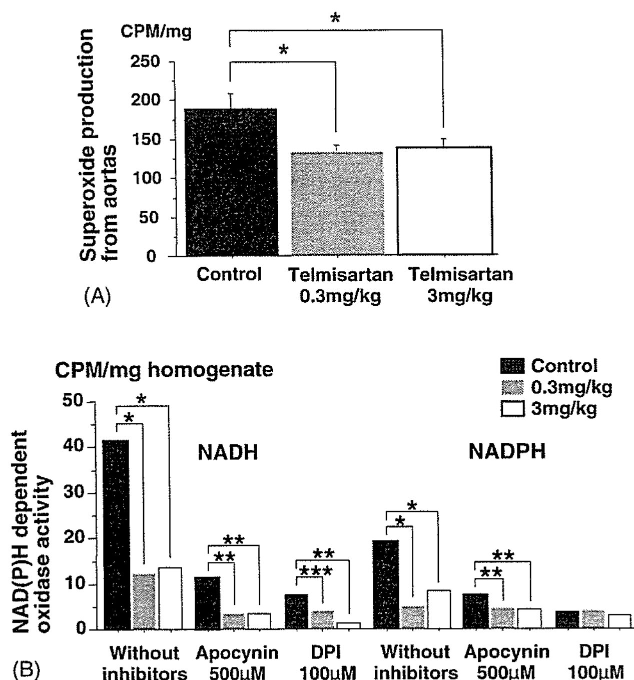

Fig. 3. (A) Effect of telmisartan on superoxide production from whole aortas using lucigenin-enhanced chemiluminescence. Aortic rings were incubated with the Cu–Zn superoxide dismutase inhibitor and vascular superoxide levels were measured by chemiluminescence with 10 μM lucigenin as described in Section 2. The counts by a luminometer were corrected by vessel dry weights. Results were expressed as mean ± S.E.M. ($n = 8$ per group). $^{*}P < 0.05$ vs. control. (B) Effect of telmisartan on NAD(P)H dependent oxidase activity of aorta homogenates using lucigenin-enhanced chemiluminescence. Aorta was homogenated and vascular NAD(P)H dependent oxidase activity was measured by use of chemiluminescence with 250 μM lucigenin in the presence of 100 μM NAD(P)H. NAD(P)H dependent oxidase activity was measured with 500 μM apocynin or 100 μM diphenylene iodinium (DPI) as described in Section 2. Results are expressed as mean ± S.E.M. of counts by luminometer in each group ($n = 8$ per group). $^{*}P < 0.0001$ vs. control; $^{**}P < 0.001$ vs. control; $^{***}P < 0.05$ vs. control.

lucigenin-enhanced CL in both control and telmisartan treatment groups.

3.5. In situ superoxide production in the vessel wall of aorta

In situ superoxide production was measured using DHE oxidative fluorescent microtopography. Ethidium fluorescence was detected throughout all layers of the vessel wall and both doses (0.3 and 3 mg/kg) of telmisartan significantly suppressed the staining (Fig. 4A). We next focused on the vascular superoxide production in the endothelial cells by measuring the ethidium fluorescence particularly on the luminal side of the internal elastic lamina. Endothelial ethidium fluorescence in 0.3 mg/kg group was decreased by 30% compared with control group and by 40% in 3 mg/kg group (Fig. 4B). These results indicated that telmisartan decreased superoxide production from the vessel wall, particularly from endothelial cells.

3.6. 8-iso-PGF2α and SAA levels

8-*iso*-PGF2α level was measured as an indicative marker of systemic oxidative stress. 8-*iso*-PGF2α levels from both urine (Fig. 5A) and plasma samples (Fig. 5B) were significantly decreased with telmisartan treatment compared with control group. On the other hand, SAA levels did not change by telmisartan (Fig. 5C).

4. Discussion

In the present study, we demonstrated that telmisartan suppressed the atherosclerotic lesion formation in apoE-KO mice. The suppressive effect was detected by 0.3 mg/kg telmisartan, which did not change systolic blood pressure, and further suppression occurred by 3 mg/kg telmisartan. As the mechanism of the drug's anti-atherogenic action, we focused on the effects for oxidative states in vivo and in vitro. Telmisartan reduced MDA- and HNE-immunostained areas compared with control group. Telmisartan suppressed superoxide production from the vessel wall via reducing NAD(P)H dependent oxidase activity. Telmisartan also reduced 8-*iso*-PGF2α levels in urine and plasma samples, which are one of indices of systemic oxidative stress. These inhibitory effects on oxidative stress were associated with suppression of atherosclerotic lesion formation.

Several animal studies demonstrated that ARB showed anti-atherogenic effects besides its effect on blood pressure [10–13]. Our finding is in agreement with the results of Hayak et al. and Dol et al., in which ARB reduced atherosclerotic lesion formation in apoE-KO mice via decreased chemokine expression and macrophage accumulation, and the inhibition of LDL oxidation [11,12]. In the present study, we demonstrated that telmisartan reduced atherosclerotic lesion formation in association with the suppression of oxidative stress via the inhibition of NAD(P)H oxidase activity.

Ang II stimulation has been reported to produce ROS from various vascular cell types [2–4]. ROS from the vessel wall are thought to play critical roles in atherogenesis. ROS induce the expression of adhesion molecules and chemokines, accelerate the formation of atherosclerotic plaque, increase matrix metalloprotease production and cause the vulnerable changes of fibrous cap [20].

In the present study, we clearly demonstrated that telmisartan suppressed superoxide production from the vessel wall assessed by lucigenin-enhanced CL method. This action was independent of the blood pressure lowering effect. We also revealed the inhibitory action of telmisartan on superoxide production by DHE staining. Telmisartan suppressed superoxide signals in all layers of aortas, particularly in the endothelium. We next focused on NAD(P)H oxidase to clarify the mechanisms of suppression of superoxide production. Superoxide anion is produced via the activation of NAD(P)H oxidase in vessel wall cells and plays an important role as the intracellular transmission factor in the Ang