厚生労働科学研究費補助金

循環器疾患等生活習慣病対策総合研究事業

動脈硬化性疾患の発症予知・進展予防に関する研究

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主任研究者

沢　村　達　也

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総括研究報告

厚生労働科学研究費補助金（循環器疾患等生活習慣病対策総合研究事業）
総括研究報告書

動脈硬化性疾患の発症予知・進展予防に関する研究

主任研究者　沢村　達也　国立循環器病センター研究所　部長

研究要旨　これまでに確立してきた血中のapoB含有LOX-1リガンド、および可溶型LOX-1測定法を用い、これらの値、更には、LOX-1とそのリガンドの相互作用を反映するパラメータと考えられるLOX Index（両者の積）を算出し、これらの心血管病バイオマーカーとしての有用性を検討した。具体的には、①血中LOX-1リガンドおよび可溶型LOX-1測定による心血管病予後予知（前向き研究）、②高脂血症患者におけるスタチン投与の血中LOX-1リガンドへの影響（介入試験）の研究を中心として進めた。その結果、①LOX-1リガンドの第4四分位の相対危険度は脳卒中・脳梗塞・冠動脈疾患いずれにおいても最も高く、脳卒中・脳梗塞においては有意な上昇となった。更に、LOX Indexの第4四分位における相対危険度はいずれにおいても有意に上昇していた。以上の結果より、LOX Indexが動脈硬化性疾患発症を予知する全く新規なバイオマーカーであることが明らかとなった。②薬物治療歴のない高脂血症患者へPitavastatinを6カ月投与したところ、血中LDLの低下とともに、LOX-1リガンドの有意な低下が観察された。興味深いことに、LDLの低下とLOX-1リガンド量の低下には有意な相関はなく、スタチンの多面的な動脈硬化性疾患抑制機序にはLOX-1リガンド量の低下も重要であることが考えられた。

分担研究者
藤田佳子　国立循環器病センター研究所　室員
木村　剛　京都大学大学院医学研究科　教授

A．研究目的

動脈硬化およびその結果として起こる虚血性心疾患は多因子病である。現状では、その危険因子のひとつである高脂血症、特に高コレステロール血症に対する治療薬として、スタチンによる治療が重用されている。一方で、やはり危険因子対策として肥満対策が政策的に行われつつある。コレステロールや肥満の動脈硬化危険因子としての重要性は明らかであるが、それだけがすべてではない事も論を待たない。例えば、コレステロール値が正常でも、酸化 LDL が高値の場合には虚血性心疾患を発症しやすい。したがって、酸化 LDL 自体が動脈硬化性疾患の危険因子である。我々も酸化 LDL 受容体 LOX-1 の研究を通じて、この分子が動脈硬化、心筋梗塞、バルーン傷害後血管再狭窄、炎症などさまざまな病態に関与する事を見出した。本研究では、これまで小動物で明らかにしてきた LOX-1 の研究成果に基づき、LOX-1 機能抑制が実際にヒトで、動脈硬化関連疾患の評価や抑制に効果があることを検証する。

一方、LOX-1 のさまざまなリガンドが明らかになり、酸化 LDL が病態悪化を招くのは、LOX-1 のような病的作用を持つ分子が作用を媒介している事も一因と考えられた。このような成果に鑑み、近年見出され、まだ作用機構が不明な危険因子の作用機序を明らかにする。これは、動脈硬化性疾患の全貌を明らかにし、新しい側面からの診断・治療法を開発するために重要と考えられる。

今年度はこれまでに確立してきた血中のapoB含有LOX-1リガンド、および可溶型LOX-1測定法を用い、これらの値、更には、LOX-1とそのリガンドの相互作用を反映するパラメータと考えられるLOX Index（両者の積）を算出し、これらの心血管病バイオマーカーとしての有用性を検討した。具体的には、①血中LOX-1リガンドおよび可溶型LOX-1測定による心血管病予後予知（前向き研究）、②高脂血症患者におけるスタチン投与の血中LOX-1リガンドへの影響（介入試験）の研究を中心として進めた。

B、C．研究方法・結果

①血中LOX-1リガンドおよび可溶型LOX-1測定による心血管病予後予知（前向き研究）

我々はこれまでに、血中のapoB含有LOX-1リガンド、および可溶型LOX-1 (sLOX-1)測定系を確立した。本研究ではこれらの他に、LOX-1とそのリガンドの相互作用を反映するパラメータと考えられる『LOX Index (LOX-1リガンド×sLOX-1)』を算出し、これらの心血管病バイオマーカーとしての有用性を検討した。

吹田研究は都市部一般市民を対象とした心血管病の発症および死亡をエンドポイントとしたコホート研究である。今回、平成6年に吹田住民健診を受診した2437名の血清のLOX-1リガンド量およびsLOX-1濃度を測定し、前向き研究を行った。全症例の脳卒中、心筋梗塞の発症を追跡調査し（平均11年間）、これらパラメータと疾患の発症との関連を検討した（コホート解析）。追跡期間の間、冠動脈疾患（68名）、脳卒中（91名、うち脳梗塞60名）の発症が観察された。LOX-1リガンド濃度、sLOX-1濃度およびLOX Indexは四分位に分け、第1四分位を基準にして比例ハザードモデルを利用して相

対危険度を求めた。昨年度はロジスティック解析を行ったが、本年度はより精度の高いこのモデルで解析を行った。

LOX-1リガンドの第4四分位の相対危険度は脳卒中(2.09)、脳梗塞（3.11)、冠動脈疾患（1.82）いずれにおいても最も高く、脳卒中・脳梗塞においては有意な上昇となった。sLOX-1においては有意な変化は認められなかった(1.03～2.13)。更にLOX Indexで評価した場合、脳梗塞の相対危険度は第2～4四分位のいずれも第1四分位と比べて非常に高かった(3.39, 3.15, 3.23)。また、冠動脈疾患の相対危険度もLOX Indexの第4四分位では2.09と有意に上昇していた。

②高脂血症患者におけるスタチン投与の血中LOX-1リガンドへの影響（介入研究）

スタチンは高コレステロール血症を示していない患者においても冠動脈疾患の発症を有意に低下させることから、LDLコレステロールの低下作用だけでは説明のつかない多面的な作用機序を有すると考えられている。Pitavastatinを始めとするいくつかのスタチンは抗酸化能を持ち、また、スタチンのLDL受容体増加作用によるLDLの代謝回転の亢進と相乗的に働き、LDLの酸化的修飾を抑制する可能性がある。今回、薬物治療歴のない25名の高脂血症患者へPitavastatinを6カ月投与し、薬物投与前後での血中LOX-1リガンド濃度の変化を測定した。

その結果、Pitavastatin投与により血中のLDLが低下するとともに、apoB含有**LOX-1リガンド量の有意な低下**が観察された（p<0.0001）。また、興味深いことに、LDLの低下とLOX-1リガンド量の低下には有意な相関は認められなかった。

D．考察

①今回の**前向き研究**により、LOX-1リガンドの高値が、特に脳梗塞発症のリスクと関連することが明らかになった。LOX-1リガンドは酸化LDLと関連した指標であるが、酸化LDLとして測定される場合でも、これまでに、前向き研究でこのような検討が報告されたことはなく、世界で初めて得られた結果である。さらに、LOX Index高値は冠動脈疾患のリスクファクターであり、LOX Index低値は脳梗塞の保護的因子（少しでも高いと危険であるという意味）であることが明らかとなった。このように、**LOX Indexが動脈硬化性疾患発症を予知する全く新しいバイオマーカー**であることが明らかとなった。これらの解析に加えて、昨年、我々は心血管病の危険因子として最近注目されているCRPがLOX-1のリガンドであることを明らかにしたが、CRPの測定も同じ対象で行い、解析を進めている。

②今回の研究の結果、LDL低下作用とは独立してスタチン投与によりapoB含有LOX-1リガンド量が低下することが明らかとなった。このことは、スタチンの多面的な動脈硬化性疾患抑制機序には、LOX-1リガンド量の低下も重要である可能性が考えられた。

E．結論

LOX-1リガンドおよび血中sLOX-1濃度の測定系を立ち上げ、これらの値並びに、LOX Indexという全く新しい心血管病のバイオマーカーを確立した。今後はこれらのバイオマーカーの他の因子との交互作用の検討や、より幅広い対象者において、これらのマーカーの予知・診断因子としての意義の検討や、LOX Index高値の人への介入試験を行い、疾患予防効果があるかどうかの検討を行いたい。

F．健康危険情報

該当なし

G．研究発表

1．論文発表

1) Eichhorn, B., Muller, G., Leuner, A., Sawamura, T., Ravens, U. and Morawietz, H.: Impaired vascular function in small resistance arteries of LOX-1 overexpressing mice on high-fat diet. Cardiovasc Res, 82:493-502, 2009.

2) Iwamoto, S., Nishimichi, N., Tateishi, Y., Sato, Y., Horiuchi, H., Furusawa, S., Sawamura, T. and Matsuda, H.: Generation and characterization of chicken monoclonal antibodies against human LOX-1. MAbs, 1:357-363, 2009.

3) Inomata, Y., Fukushima, M., Hara, R., Takahashi, E., Honjo, M., Koga, T., Kawaji, T., Satoh, H., Takeya, M., Sawamura, T. and Tanihara, H.: Suppression of choroidal neovascularization in lectin-like oxidized low-density lipoprotein receptor type 1-deficient mice. Invest Ophthalmol Vis Sci, 50:3970-3976, 2009.

4) Ogura, S., Kakino, A., Sato, Y., Fujita, Y., Iwamoto, S., Otsui, K., Yoshimoto, R. and Sawamura, T.: LOX-1: the multifunctional receptor underlying cardiovascular dysfunction. Circ J, 73:1993-1999, 2009.

5) Sakamoto, N., Ishibashi, T., Sugimoto, K., Sawamura, T., Sakamoto, T., Inoue, N., Saitoh, S., Kamioka, M., Uekita, H., Ohkawara, H., Suzuki, K., Teramoto, T., Maruyama, Y. and Takeishi, Y.: Role of LOX-1 in monocyte adhesion-triggered redox, Akt/eNOS and Ca2+ signaling pathways in endothelial cells. J Cell Physiol, 220:706-715, 2009.

6) Sankaralingam, S., Xu, H., Jiang, Y., Sawamura, T. and Davidge, S.T.: Evidence for increased methylglyoxal in the vasculature of women with preeclampsia: role in upregulation of LOX-1 and arginase. Hypertension, 54:897-904, 2009.

7) Schaeffer, D.F., Riazy, M., Parhar, K.S., Chen, J.H., Duronio, V., Sawamura, T. and Steinbrecher, U.P.: LOX-1 augments oxLDL uptake by lysoPC-stimulated murine macrophages but is not required for oxLDL clearance from plasma. J Lipid Res, 50:1676-1684, 2009.

8) Sugimoto, K., Ishibashi, T., Sawamura, T., Inoue, N., Kamioka, M., Uekita, H., Ohkawara, H., Sakamoto, T., Sakamoto, N., Okamoto, Y., Takuwa, Y., Kakino, A., Fujita, Y., Tanaka, T., Teramoto, T., Maruyama, Y. and Takeishi, Y.: LOX-1-MT1-MMP axis is crucial for RhoA

and Rac1 activation induced by oxidized low-density lipoprotein in endothelial cells. Cardiovasc Res, 84:127-136, 2009.
9) Zushi, S., Akagi, M., Kishimoto, H., Teramura, T., Sawamura, T. and Hamanishi, C.: Induction of bovine articular chondrocyte senescence with oxidized low-density lipoprotein through lectin-like oxidized low-density lipoprotein receptor 1. Arthritis Rheum, 60:3007-3016, 2009.
10) Fujita, Y., Kakino, A., Harada-Shiba, M., Sato, Y., Otsui, K., Yoshimoto, R. and Sawamura, T.: C-reactive protein uptake by macrophage cell line via class-A scavenger receptor. Clin Chem, 56:478-481, 2010.
11) Nakano, A., Inoue, N., Sato, Y., Nishimichi, N., Takikawa, K., Fujita, Y., Kakino, A., Otsui, K., Yamaguchi, S., Matsuda, H. and Sawamura, T.: LOX-1 mediates vascular lipid retention under hypertensive state. J Hypertens, 28:1273-1280, 2010.
12) Takaya, T., Wada, H., Morimoto, T., Sunagawa, Y., Kawamura, T., Takanabe-Mori, R., Shimatsu, A., Fujita, Y., Sato, Y., Fujita, M., Kimura, T., Sawamura, T. and Hasegawa, K.: Left ventricular expression of lectin-like oxidized low-density lipoprotein receptor-1 in failing rat hearts. Circ J, 74:723-729, 2010.
13) Inoue, N., Okamura, T., Kokubo, Y., Fujita, Y., Sato, Y., Nakanishi, M., Yanagida, K., Kakino, A., Iwamoto, S., Watanabe, M., Ogura, S., Otsui, K., Matsuda, H., Uchida, K., Yoshimoto, R. and Sawamura, T.: LOX index, a novel predictive biochemical marker for coronary heart disease and stroke. Clin Chem, 56:550-558, 2010.

2. 学会発表

沢村達也「酸化LDL受容体LOX-1と心血管病」

徳島大学セミナー　淡路島　2010月1月29-30日

H. 知的財産権の出願・登録状況

該当なし

分担研究報告

厚生労働科学研究費補助金（循環器疾患等生活習慣病対策総合研究事業）
分担研究報告書

動脈硬化危険因子の臨床的意義の検討に関する研究

分担研究者　木村　剛　京都大学大学院医学研究科　循環器内科学　教授

研究要旨　急性冠症候群をはじめとする虚血性心疾患による死亡は、我が国でも増加の一途をたどっており、その危険因子を明らかにし、発症予知の精度を高めることは極めて重要である。我々は、動脈硬化性疾患の危険因子として、脂質、特に酸化脂質が重要であることを、1）坑酸化剤プロブコールが動脈硬化進展を抑制すること、2）酸化LDL、その構成脂質であるリゾフォスファチジルコリンが催動脈硬化性接着分子、増殖因子などの発現を誘導すること、などを明らかにすることで提唱してきた。その流れは、本研究主任研究者との共同研究による3）新規酸化LDL受容体LOX-1の同定、更に４）可溶型LOX-1が急性冠症候群超急性期のマーカーになる可能性の提唱、につながっている。本研究では、1）血中可溶型LOX-1濃度の臨床的意義を更に追求すること、2）可溶型LOX-1産生の分子機構を解明すること、3）LOX-1の不安定プラーク画像診断における有用性の検討を3本柱として進めた。平成21年度は、1）に関連して、血中可溶型LOX-1濃度のさらに鋭敏な測定を可能にする新規測定系の開発、および2）に関連して、LOX-1のシェディングの増強因子ナルディライジンの生理的意義の解明、を目的として研究した。

A．研究目的

1）我々は、sLOX-1が急性冠症候群発症急性期の鋭敏なマーカーであることを報告してきた。ところが、これまで用いてきた測定系は、健常者の血中sLOX-1測定には感度が十分でなかったため、sLOX-1のさらなる臨床応用を目的に、より感度の高いsLOX-1測定系の開発に取り組む。

２）我々は、sLOX-1が膜結合型LOX-1の細胞外ドメインシェディングにより産生され、同プロセスが炎症性サイトカインによって誘導されることを明らかにした。一方我々は、独自に同定した細胞外ドメインシェディング活性化因子ナルディライジンが、LOX-1のシェディングも増強することを確認した。今回、ナルディライジンの欠損マウスを作製し、同分子の生理作用を明らかにし、ひいてはLOX-1シェディングの生理的意義を解明する。

B．研究方法

1）sLOX-1を抗原として、新たなモノクローナル抗体を作製し、同抗体の組み合わせによる、サンドイッチELISA（発光免疫測定法）システムを構築する。

2）ナルディライジン欠損マウスを遺伝子ターゲティング法にて作製し、その表現型を解析する。

C．研究結果

1）新たに9種類のLOX-1抗体を作製した。これらの抗体とsLOX-1の解離定数は0.12-1.32 nMであった。抗体の組み合わせを検討し、検出感度8 pg/mLという、高感度測定系（発光免疫測定法）の開発に成功した（Nakamura et.al. J Pharm Biomed Anal. 2010）。

2）ナルディライジン（NRDc）はADAM10によるLOX-1シェディングを増強した。ナルディライジンの生物学的作用を明らかにするため、ナルディライジン欠損マウスを作製したところ、同ホモ接合体（NRDc-/-）はほぼメンデルの法則にそって誕生したが、約70%が48時間以内に死亡した。NRDc-/-の出生時体重はNRDc+/+の70%程度で、生後の成長遅延も認めたが、生き残ったNRDc-/-は摂食能、運動能に一見問題なく元気に発育した。しかしながら、異常反射が陽性であったこと、生後3ヶ月前後から頚を大きく上下させるてんかん様不随意運動を呈したことから、中枢神経系の異常を疑い詳細に検討したところ、NRDcがニューレギュリンのシェディングを介して、神経軸索・髄鞘形成を司ることを明らかにし、NRDcが個体レベルにおいても、欠くべからざるシェディング調節因子であることを明らかにした（Ohno et.al. Nat. Neurosci. 2009）。

D．考察

急性冠症候群急性期にsLOX-1が高値であった症例の予後が悪かったことから、sLOX-1がプラークの不安定性のマーカーであることが示唆された。今後sLOX-1が発症のリスクを予見できるかを、今回開発した高感度測定系を用いて大規模前向き試験により明らかにしたい。また、近年肥満（内臓脂肪の蓄積）とインシュリン抵抗性を基盤とし、複数の動脈硬化の危険因子（高脂血症、耐糖能障害、高血圧）が個人に集積する病態が、メタボリックシンドロームとして提唱され、急性冠症候群のハイリスクグループとして注目されている。同シンドローム症例でのsLOX-1値も、同様に新規測定系にて検討する予定である。さらに、sLOX-1産生に重要である可能性が示唆されたナルディライジンの欠損マウスを用いて、sLOX-1シェディングにおけるナルディライジンの重要性を明らかにしたい。

E．結論
sLOX-1の超高感度測定系の開発に成功した。ナルディライジン欠損マウスの作製に成功し、同分子が個体レベルにおいても、欠くべからざるシェディング調節因子であることを明らかにした。

F．健康危険情報
該当なし

G．研究発表
1．論文発表

1) Nakagawa Y, Nobuyoshi M, Yamaguchi T, Meguro T, Yokoi H, Kimura T, Hosoda S, Kanmatsuse K, Matsumori A, Sasayama S. Efficacy of abciximab for patients undergoing balloon angioplasty: data from Japanese evaluation of c7E3 Fab for elective and primary PCI organization in randomized trial (JEPPORT). Circ J. 73(1):145-151, 2009.
2) Furukawa Y, Ehara N, Taniguchi R, Haruna Y, Ozasa N, Saito N, Doi T, Hoshino K, Tamura T, Shizuta S, Abe M, Toma M, Morimoto T, Teramukai S, Fukushima M, Kita T, Kimura T. Coronary risk factor profile and prognostic factors for young japanese patients undergoing coronary revascularization Circ J. 73(8): 1459-65, 2009.
3) Kimura T, Morimoto T, Nakagawa Y, Tamura T, Kadota K, Yasumoto H, Nishikawa H, Hiasa Y, Muramatsu T, Meguro T, Inoue N, Honda H, Hayashi Y, Miyazaki S, Oshima S, Honda T, Shiode N, Namura M, Sone T, Nobuyoshi M, Kita T, Mitsudo K. Antiplatelet therapy and stent thrombosis after sirolimus-eluting stent implantation. Circulation. 119(7): 987-995, 2009.
4) Nishiyama K, Horiguchi M, Sakata R, Okabayashi H, Yamanaka K, Ushijima T, Nishiwaki N, Okada Y, Tanaka K, Shimamoto M, Asai T, Aota M, Tahata T, Matsuda K, Nakajima H, Kawasuji M, Komiya T, Furukawa Y, Nakagawa Y, Ehara N, Taniguchi R, Haruna Y, Shizuta S, Doi T, Fukushima M, Kita T, Kimura T. Temporal pattern of strokes after on-pump and off-pump coronary artery bypass graft surgery. Ann Thorac Surg. 87(6): 1839-44, 2009.
5) Abe M, Kimura T, Morimoto T, Furukawa Y, Kita T. Incidence of and risk factors for contrast-induced nephropathy after cardiac catheterization in Japanese patients. Circ J. 73(8): 1518 -1522, 2009.
6) Jinnai T, Horiuchi H, Makiyama T, Tazaki J, Tada T, Akao M, Ono K, Hoshino K, Naruse Y, Takahashi K, Watanabe H, Kita T, Kimura T. The impact of CYP2C19 polymorphisms on the antiplatelet effect of clopidgrel in an actual clinical setting in Japan. Circ.J. 73(8): 1498-503, 2009.
7) Hiro T, Kimura T, Morimoto T, Miyauchi K, Nakagawa Y, Yamagishi M, Ozaki Y, Kimura K, Saito S, Yamaguchi T, Daida H, Matsuzaki M, for the JAPAN-ACS Investigators. Effect of intensive statin therapy on regression of coronary atherosclerosis in patients with acute coronary syndrome: A multi-center randomized trial evaluated by volumetric intravascular ultrasound using pitavastatin versus atorvastatin (JAPAN-ACS Study). J Am Coll Cardiol. 54:293-302(4), 2009.
8) Toyofuku M, Kimura T, Morimoto T, Hayashi Y, Ueda H, Kawai K, Nozaki Y, Hiramatsu S, Miura A, Yokoi Y, Toyoshima S, Nakashima H, Haze K, Tanaka M, Take S, Saito S, Isshiki T, Mitsudo K, on behalf of the j-Cypher Registry InvestigatorsThree- year outcomes after sirolimus-eluting stent implantation forunprotected left main coronary artery disease: insights from the j-Cypher registry. Circulation . 120: 1866-1874, 2009.
9) Kimura T, Isshiki T, Hayashi Y, Oshima S,Namura M, Nakashima H, Kawai K, Sone T, Tatami R, Meguro T, Nobuyoshi M, Mitsudo K. Incidence and outcome of surgical procedures after sirolimus-eluting stent implantation: areport from the j-Cypher registry. Cardiovasc Interv and Ther. 25: 29, 2009
10) Horie T, Ono K, Nishi H, Iwanaga Y, Nagao K, Kinoshita M, Kuwabara Y, Takanabe R, Hasegawa K, Kita T, Kimura T. MicroRNA-133 regulates the expression of GLUT4 by targeting KLF15 and is involved in metabolic control in cardiac myocytes. Biochem Biophys Res Commun. 389(2): 315-20, 2009.
11) Nishio Y, Makiyama T, Itoh H, Sakaguchi T, Ohno S, Gong YZ, Yamamoto S, Ozawa T, Ding WG, Toyoda F, Kawamura M, Akao M, Matsuura H, Kimura T, Kita T, Horie M. D85N, a KCNE1 polymorphism, is a disease-causing gene variant in long QT syndrome. J Am Coll Cardiol.54(9): 812-9, 2009.
12) Shirakawa R, Fukai S, Kawato M, Higashi T, Kondo H, Ikeda T, Nakayama E, Okawa K, Nureki O, Kimura T, Kita T, Horiuchi H. Tuberous sclerosis tumor suppressor complex-like complexes act as GTPase-activating proteins for Ral GTPases. J Biol Chem. 284(32): 21580-8, 2009.
13) Kojima Y, Ono K, Inoue K, Takagi Y, Kikuta K, Nishimura M, Yoshida Y, Nakashima Y, Matsumae H, Furukawa Y, Mikuni N, Nobuyoshi M, Kimura T, Kita T, Tanaka M. Progranulin expression in advanced human atherosclerotic plaque. Atherosclerosis. 206(1): 102-8. 2009.
14) Mima A, Toma M, Matsubara T, Shiota F, Iehara N, Abe H, Nagai K, Takahashi T, Matsuura M, Murakami T, Kishi S, Araoka T, Kishi F, Kondo N, Shigeta R, Yoshikawa K, Kimura T, Kita T, Doi T, Fukatsu A. Angio-embolization of renal artery pseudoaneurysm after renal biopsy: a case report. Ren Fail. 31(8): 753-5, 2009.
15) Nishi H, Ono K, Iwanaga Y, Horie T, Nagao K, Takemura G, Kinoshita M, Kuwabara Y, Mori RT, Hasegawa K, Kita T, Kimura T. MicrorRNA-15b modulates cellular atp levels and degenerates mitochondria via arl2 in neonatal rat cardiac myocytes. J Biol Chem. 285(7):4920-30, 2009.
16) Morino Y, Kimura T, Hayashi Y, Muramatsu T, Ochiai M, Noguchi Y, Kato K, Shibata Y, Hiasa Y, Doi O, Yamashita T, Morimoto T, Abe M, Hinohara T, Mitsudo K; J-CTO Registry Investigators. In-hospital outcomes of contemporary percutaneous coronary intervention in patients with chronic total occlusion insights from the J-CTO Registry (Multicenter CTO Registry in Japan). JACC Cardiovasc Interv. 2010 Feb;3(2):143-51.
17) Nakamura M, Ohta H, Kume N, Hayashida K, Tanaka M, Mitsuoka H, Kaneshige T, Misaki S, Imagawa K, Shimosako K, Ogawa N, Kita T, Kominami G. Generation of monoclonal antibodies against a soluble form of lectin-like oxidized low-density lipoprotein receptor-1 and development of a sensitive

chemiluminescent enzyme immunoassay. J Pharm Biomed Anal. 2010; 51:158-63.
18) Ohno M, Hiraoka Y, Matsuoka T, Tomimoto H, Takao K, Miyakawa T, Oshima N, Kiyonari H, Kimura T, Kita T, and Nishi E. Nardilysin Regulates Axonal Maturation and Myelination in the Central and Peripheral Nervous System. Nat. Neurosci. 2009; 12: 1506-1513.

2.学会発表

1) 14th Annual Angioplasty Summit TCT Asia Pacific 2009, April 22-24, 2009, Seoul, Korea Kimura T：Antiplatelet Therapy and Stent Thrombosis After Sirolimus-Eluting Stent Implantation
2) The 5th Annual Scientific Conference at the Cardiovascular Center, NTUH Yun-Lin Branch, August 1-3, 2009, Taiwan Kimura T：Time Dependent Differences in the Demographics and Clinical Outcome of Stent Thrombosis of Sirolimus-eluting Stent: Final result from the RESTART (REgistry of Stent Thrombosis for review And Re-evaluaTion) registry.
3) ESC Congress 2009, August 29-September 2, 2009, Barcelona, Spain Kimura T: 1) Antiplatelet therapy discontinuation and stent thrombosis of sirolimus-eluting stent: observation from the RESTART (REgistry of Stent Thrombosis for review And Re-evaluaTion),.2)Time dependent differences in the demographics and clinical outcome of stent thrombosis of sirolimus-eluting stent: observations from the RESTART.
4) TCT 2009, September 21 - 26, 2009, San Francisco, US, Kimura T: DES Safety; Insights from Clinical Trials
5) AHA Scientific Sessions 2009, November 14-18, 2009, Florida, US. Kimura T: Incidence and Outcome of Surgical Procedures After Sirolimus-eluting Stent Implantation
6) 第73回日本循環器学会総会・学術集会、3.20-22 大阪、木村　剛：１）ランチョンセミナーLS-20、3.20　急性冠症候群患者の最新の治療戦略、２）教育セッション・ライブデモンストレーション ビデオライブで見るリスクとベネフィットを考えた治療戦略、3.20　DES血栓症、３）ファイアサイドセミナー20、3.21　冠動脈疾患と抗血小板療法
7) 第１８回日本心血管インターベンション治療学会学術集会、6.25-26、愛知、木村　剛　１）全国のステント血栓症レジストリー（RESTART）からの知見－臨床データから－、２）JSIC/JACCT歴代理事長による統合記念講演－日本のPCIにおける将来への期待－、３）PCIと抗血小板療法　Role of long-term aggressive anti-thrombotic therapy in Japanese patients with coronary artery disease、４）CTO PCI治療の適応－病変選択と患者選択（日本の現状）
8) 第１４回　日本冠動脈外科学会学術大会、7.16、熊本、木村　剛：薬剤溶出性ステント（DES）を用いたPCI：最近の動向
9) 第13回日本心不全学会学術集会、10.30～11.1、福岡　木村　剛：J-Cypher Registry Update

H．知的財産権の出願・登録状況

なし

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻	ページ	出版年
Eichhorn, B., Muller, G., Leuner, A., Sawamura, T., Ravens, U. and Morawietz, H.	Impaired vascular function in small resistance arteries of LOX-1 overexpressing mice on high-fat diet.	Cardiovasc Res	82	493-502	2009
Iwamoto, S., Nishimichi, N., Tateishi, Y., Sato, Y., Horiuchi, H., Furusawa, S., Sawamura, T. and Matsuda, H.	Generation and characterization of chicken monoclonal antibodies against human LOX-1.	MAbs	1	357-363	2009
Inomata, Y., Fukushima, M., Hara, R., Takahashi, E., Honjo, M., Koga, T., Kawaji, T., Satoh, H., Takeya, M., Sawamura, T. and Tanihara, H.	Suppression of choroidal neovascularization in lectin-like oxidized low-density lipoprotein receptor type 1-deficient mice.	Invest Ophthalmol Vis Sci	50	3970-3976	2009
Ogura, S., Kakino, A., Sato, Y., Fujita, Y., Iwamoto, S., Otsui, K., Yoshimoto, R. and Sawamura, T.	LOX-1: the multifunctional receptor underlying cardiovascular dysfunction.	Circ J	73	1993-1999	2009
Sakamoto, N., Ishibashi, T., Sugimoto, K., Sawamura, T., Sakamoto, T., Inoue, N., Saitoh, S., Kamioka, M., Uekita, H., Ohkawara, H., Suzuki, K., Teramoto, T., Maruyama, Y. and Takeishi, Y.	Role of LOX-1 in monocyte adhesion-triggered redox, Akt/eNOS and Ca2+ signaling pathways in endothelial cells.	J Cell Physiol	220	706-715	2009
Sankaralingam, S., Xu, H., Jiang, Y., Sawamura, T. and Davidge, S.T.	Evidence for increased methylglyoxal in the vasculature of women with preeclampsia: role in upregulation of LOX-1 and arginase.	Hypertension	54	897-904	2009
Schaeffer, D.F., Riazy, M., Parhar, K.S., Chen, J.H., Duronio, V., Sawamura, T. and Steinbrecher, U.P.	LOX-1 augments oxLDL uptake by lysoPC-stimulated murine macrophages but is not required for oxLDL clearance from plasma.	J Lipid Res	50	1676-1684	2009
Sugimoto, K., Ishibashi, T., Sawamura, T., Inoue, N., Kamioka, M., Uekita, H., Ohkawara, H., Sakamoto, T., Sakamoto, N., Okamoto, Y., Takuwa, Y., Kakino, A., Fujita, Y., Tanaka, T., Teramoto, T., Maruyama, Y. and Takeishi, Y.	LOX-1-MT1-MMP axis is crucial for RhoA and Rac1 activation induced by oxidized low-density lipoprotein in endothelial cells.	Cardiovasc Res	84	127-136	2009
Zushi, S., Akagi, M., Kishimoto, H., Teramura, T., Sawamura, T. and Hamanishi, C.	Induction of bovine articular chondrocyte senescence with oxidized low-density lipoprotein through lectin-like oxidized low-density lipoprotein receptor 1.	Arthritis Rheum	60	3007-3016	2009

Kimura, T., Morimoto, T., Nakagawa, Y., Tamura, T., Kadota, K., Yasumoto, H., Nishikawa, H., Hiasa, Y., Muramatsu, T., Meguro, T., Inoue, N., Honda, H., Hayashi, Y., Miyazaki, S., Oshima, S., Honda, T., Shiode, N., Namura, M., Sone, T., Nobuyoshi, M., Kita, T., Mitsudo, K.	Antiplatelet therapy and stent thrombosis after sirolimus-eluting stent implantation	Circulation	119	987-995	2009
Nishiyama, K., Horiguchi, M., Sakata, R., Okabayashi, H., Yamanaka, K., Ushijima, T., Nishiwaki, N., Okada, Y., Tanaka, K., Shimamoto, M., Asai, T., Aota, M., Tahata, T., Matsuda, K., Nakajima, H., Kawasuji, M., Komiya, T., Furukawa, Y., Nakagawa, Y., Ehara, N., Taniguchi, R., Haruna, Y., Shizuta, S., Doi, T., Fukushima, M., Kita, T., Kimura, T.	Temporal pattern of strokes after on-pump and off-pump coronary artery bypass graft surgery.	Ann Thorac Surg	87	1839-44	2009
Hiro, T., Kimura, T., Morimoto, T., Miyauchi, K., Nakagawa, Y., Yamagishi, M., Ozaki, Y., Kimura, K., Saito, S., Yamaguchi, T., Daida, H., Matsuzaki, M., for the JAPAN-ACS Investigators	Effect of intensive statin therapy on regression of coronary atherosclerosis in patients with acute coronary syndrome: A multi-center randomized trial evaluated by volumetric intravascular ultrasound using pitavastatin versus atorvastatin (JAPAN-ACS Study)	J Am Coll Cardiol	54	293-302	2009
Toyofuku, M., Kimura, T., Morimoto, T., Hayashi, Y.,Ueda, H., Kawai, K., Nozaki, Y., Hiramatsu, S., Miura, A., Yokoi, Y., Toyoshima, S., Nakashima, H., Haze, K., Tanaka, M., Take, S., Saito, S., Isshiki, T., Mitsudo, K.,on behalf of the j-Cypher Registry Investigators	Three- year outcomes after sirolimus-eluting stent implantation forunprotected left main coronary artery disease: insights from the j-Cypher registry	Circulation	120	1866-1874	2009
Kimura, T., Isshiki, T., Hayashi, Y., Oshima, S., Namura, M., Nakashima, H., Kawai, K., Sone, T., Tatami, R., Meguro, T., Nobuyoshi, M., Mitsudo, K.	Incidence and outcome of surgical procedures after sirolimus-eluting stent implantation: areport from the j-Cypher registry	Cardiovasc Interv and Ther	25	29-39	2009
Nishio, Y., Makiyama, T., Itoh, H., Sakaguchi, T., Ohno, S., Gong, YZ., Yamamoto, S., Ozawa, T., Ding WG, Toyoda, F., Kawamura, M., Akao, M., Matsuura, H., Kimura, T., Kita, T., Horie, M.	D85N, a KCNE1 polymorphism, is a disease-causing gene variant in long QT syndrome	J Am Coll Cardiol	54	812-819	2009

Shirakawa, R., Fukai, S., Kawato, M., Higashi, T., Kondo, H., Ikeda, T., Nakayama, E., Okawa, K., Nureki, O., Kimura, T., Kita, T., Horiuchi, H.	Tuberous sclerosis tumor suppressor complex-like complexes act as GTPase-activating proteins for Ral GTPases	J Biol Chem	284	21580-21588	2009
Nishi, H., Ono, K., Iwanaga, Y., Horie, T., Nagao, K., Takemura, G., Kinoshita, M., Kuwabara, Y., Mori, RT., Hasegawa, K., Kita, T., Kimura, T.	MicrorRNA-15b modulates cellular atp levels and degenerates mitochondria via arl2 in neonatal rat cardiac myocytes	J Biol Chem	285	4920-4930	2009
Ohno, M., Hiraoka, Y., Matsuoka, T., Tomimoto, H., Takao, K., Miyakawa, T., Oshima, N., Kiyonari, H., Kimura, T., Kita, T., Nishi, E.	Nardilysin Regulates Axonal Maturation and Myelination in the Central and Peripheral Nervous System.	Nat Neurosci	12	1506-1513	2009
Fujita, Y., Kakino, A., Harada-Shiba, M., Sato, Y., Otsui, K., Yoshimoto, R. and Sawamura, T.	C-Reactive Protein Uptake by Macrophage Cell Line via Class-A Scavenger Receptor.	Clin Chem	56	478-481	2010
Nakano, A., Inoue, N., Sato, Y., Nishimichi, N., Takikawa, K., Fujita, Y., Kakino, A., Otsui, K., Yamaguchi, S., Matsuda, H. and Sawamura, T.	LOX-1 mediates vascular lipid retention under hypertensive state.	J Hypertens	28	1273-1280	2010
Takaya, T., Wada, H., Morimoto, T., Sunagawa, Y., Kawamura, T., Takanabe-Mori, R., Shimatsu, A., Fujita, Y., Sato, Y., Fujita, M., Kimura, T., Sawamura, T. and Hasegawa, K.	Left ventricular expression of lectin-like oxidized low-density lipoprotein receptor-1 in failing rat hearts.	Circ J	74	723-729	2010
Inoue, N., Okamura, T., Kokubo, Y., Fujita, Y., Sato, Y., Nakanishi, M., Yanagida, K., Kakino, A., Iwamoto, S., Watanabe, M., Ogura, S., Otsui, K., Matsuda, H., Uchida, K., Yoshimoto, R. and	LOX index, a novel predictive biochemical marker for coronary heart disease and stroke.	Clin Chem	56	550-558	2010
Nakamura, M., Ohta, H., Kume, N., Hayashida, K., Tanaka, M., Mitsuoka, H., Kaneshige, T., Misaki, S., Imagawa, K., Shimosako, K., Ogawa, N., Kita, T., Kominami, G.	Generation of monoclonal antibodies against a soluble form of lectin-like oxidized low-density lipoprotein receptor-1 and development of a sensitive chemiluminescent enzyme immunoassay.	J Pharm Biomed Anal	51	158-163	2010

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Impaired vascular function in small resistance arteries of LOX-1 overexpressing mice on high-fat diet

Birgit Eichhorn[1]*, Gregor Muller[2], Anja Leuner[2], Tatsuya Sawamura[3], Ursula Ravens[1], and Henning Morawietz[2]

[1]*Department of Pharmacology and Toxicology, Medical Faculty Carl Gustav Carus, University of Technology, Fetscherstr. 74, D-01307 Dresden, Germany;* [2]*Department of Vascular Endothelium and Microcirculation, Medical Faculty Carl Gustav Carus, University of Technology, Dresden, Germany; and* [3]*Department of Vascular Physiology, National Cardiovascular Center Research Institute, Suita, Osaka, Japan*

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Aims LOX-1 is a major vascular receptor for oxidized low-density lipoprotein (oxLDL). In this study, we analysed the impact of LOX-1 overexpression and high dietary fat intake on vascular function in small resistance arteries.

Methods and results Relaxation of mesenteric arteries was measured using a wire myograph. Compared with the control group, mice overexpressing LOX-1 on a high-fat diet (FD) had preserved vascular smooth muscle relaxation, but impaired endothelium-dependent relaxation via NO. Vascular NO availability was decreased by exaggerated formation of reactive oxygen species and decreased endothelial NO synthase expression. Endothelium-derived hyperpolarizing factor (EDHF)-mediated relaxation via cytochrome P450 metabolites was increased in LOX-1 + FD animals, but did not completely compensate for the loss of NO. Currents of calcium-activated potassium channels with large conductance (BK_{Ca} channels) were measured by the voltage-clamp method. The BK_{Ca} current amplitudes were not altered in endothelial cells, but highly increased in vascular smooth muscle cells from resistance arteries of LOX-1-overexpressing mice on FD. BK_{Ca} currents were activated by low-dose H_2O_2 and cytochrome P450 metabolites 11,12-EET and 14,15-EET as EDHF in control mice.

Conclusion LOX-1 overexpression and FD caused functional changes in endothelial and vascular smooth muscle cells of small resistance arteries.

1. Introduction

Atherosclerosis, with its clinical manifestation in cardiovascular diseases, is the major cause of death in industrialized countries. Functional changes in endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) contribute to the initiation and early progression of cardiovascular diseases like atherosclerosis.[1–3] Resistance arteries do not show morphological alterations in response to high-fat diet (FD) or oxLDL, but rather develop functional impairment.[4] Several changes in the early phase of endothelial dysfunction are associated with high plasma levels of lipoproteins. Circulating low-density lipoproteins (LDL) can be modified to oxidized LDL (oxLDL). The major receptor of oxLDL is the lectin-like oxLDL receptor-1 (LOX-1) in the vessel wall.[5–7] LOX-1 mediates endocytosis of oxLDL in ECs,[8] VSMCs, and monocytes.[9] Basal LOX-1 expression is low, but several pathophysiological conditions like hypertension, diabetes mellitus, and hyperlipidaemia and the development of atherosclerotic lesions have been linked with an increased vascular LOX-1 expression.[10] The G501C mutation in the lectin-like oxidized LDL receptor gene (LOX-1/OLR1) has been associated with the risk of myocardial infarction,[11] but not with the risk for stroke.[12] LOX-1 expression is increased in human atherosclerotic lesions (in early lesions mainly in ECs, in advanced lesions also in VSMCs and macrophages).[13] Moreover plaque formation is enhanced in coronary arteries of mice overexpressing LOX-1 against a genetic background of apolipoprotein E deficiency.[14] Cell-culture studies have shown that the endothelial generation of reactive oxygen species (ROS) by NAD(P)H oxidase complexes in response to oxLDL is mediated by LOX-1.[15] Furthermore, *in vitro* studies indicate that activation of LOX-1 also initiates a reduction in NO release.[15] However, little is known about the contribution of LOX-1 to vascular homeostasis and endothelial dysfunction in small vessels. The intact endothelium plays an important role in vascular function by synthesizing and releasing vasodilating factors.[16] Major vasodilating factors in arteries are the endothelium-derived hyperpolarizing factor (EDHF), nitric oxide (NO),

* Corresponding author. Tel: +49 351 458 6261; fax: +49 351 458 6315.
E-mail address: birgit.eichhorn@tu-dresden.de

and prostacyclin. The reduced NO availability in atherosclerosis can be mediated via decreased expression of the endothelial NO synthase (eNOS) or via inactivation of NO by ROS.[2] In addition to eNOS expression, there is also evidence of reduced eNOS activity by lack of cofactors (e.g. BH_4) and increased formation of endogenous inhibitors.[17] The impaired NO-mediated relaxation in different vessels of hypercholesterolaemic and atherosclerotic animal models or patients[18,19] can be compensated by EDHF. Several components for EDHF signalling have been proposed including electrical coupling through gap junctions, certain ROS as for instance H_2O_2, cytochrome P450 metabolites, and vascular Ca^{2+}-activated K^+ channels (K_{Ca})[20] in particular those with large conductance (BK_{Ca} channels).[21–23] Activation of BK_{Ca} channels facilitates relaxation by cell membrane hyperpolarization. Nevertheless their role in endothelial and smooth muscle dysfunction is not completely understood. Binding of oxLDL to LOX-1 was shown to modulate BK_{Ca}-channels in ECs *in vitro*.[15]

In this study, we have used mice overexpressing bovine LOX-1 that were fed an FD to examine a potential functional impairment of small resistance arteries (mesenteric arteries). Based on our experimental findings, we provide evidence that LOX-1 receptors can cause vascular dysfunction in resistance vessels.

2. Methods

2.1 Animals

Male wild-type mice (C57BL/6, WT) and mice overexpressing bovine LOX-1 in a C57BL/6 background under control of the murine preproendothelin-1 promoter (LOX-1 mice[14]), aged 8 weeks, were fed standard chow EF R/M CD88137 or FD EF R/M TD88137 (Ssniff Spezialitäten GmbH, Soest, Germany) for 10 weeks. LOX-1 mice were kindly provided by T.S., Department of Vascular Physiology, National Cardiovascular Center Research Institute Fujishirodai, Suita, Osaka, Japan. The generation and characterization of the mice has been recently described by Inoue *et al.*[14] The LOX-1 mice carry 24 copies of the transgene, resulting in an approximately eight-fold higher mRNA-expression and a marked upregulation of endothelial LOX-1 protein expression. A similar upregulation of the LOX-1 protein has been described in ECs of human carotid arteries, covering early atherosclerotic lesions.[13] Overexpression of the bovine LOX-1 was verified by PCR in mesenteric arteries (see Supplementary material online, *Figure S1*). All performed experiments are in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal research Ethics Committee of the Dresden University of Technology and the Regierungspräsidium Dresden approved the animal facilities and the experiments according to institutional guidelines and German animal welfare regulations (AZ: 24-9168.24-1-2003-13, 24D-9168.24-1/2006-16).

2.2 Serum lipid measurements

Serum lipids were measured at the Institute of Clinical Chemistry and Laboratory Medicine (University of Technology Dresden) using kits for triglycerides, cholesterol, HDLs and LDLs (Roche Diagnostics GmbH, Mannheim, Germany).

2.3 Preparation of mesenteric arteries for *in vitro* studies

Arteries (third-order branch) were dissected and maintained in Ca^{2+}-free physiological salt solution [PSS; mmol/L: NaCl 119.0; KCl 4.7; $MgSO_4$ 1.17; $NaHCO_3$ 25.0; KH_2PO_4 1.18; glucose 5.5, and ethylenediaminetetraacetic acid (EDTA) 0.027; pH 7.4].

2.4 Superoxide anions

Dissected vessels were incubated in Krebs–Henseleit buffer (mmol/L: NaCl 115.0; $NaHCO_3$ 25.0; KCl 4.0; KH_2PO_4 0.9; $MgSO_4 \times 7H_2O$ 1.1; $CaCl_2$ 2.6; glucose 5.5; pH: 7.4) for 30 min at 37°C. Lucigenin (5 μmol/L – a concentration below the threshold of redox cycling[24]) and NADPH (100 μmol/L) were dissolved in Krebs–Henseleit buffer for determination of ROS. Lucigenin solution containing additional 200 U/mL superoxide dismutase (SOD) and 380 U/mL catalase was used to examine superoxide anions and the resulting H_2O_2 formation.[25] Solution without tissue served as control. Photoemission was detected every second for 30 min in a Fluorescence Microplate Reader Fluorimeter FLUOstar OPTIMA (BMG LABTECH, Jena, Germany). The length of the blood vessels was measured with an eye piece scale (ZEISS, Jena, Germany) and used for data normalization. The increase in ROS production in animals fed with an FD was normalized as 100% of the corresponding control.

2.5 Measurement of contractile function

Small sections of mesenteric arteries (length 2 mm) were mounted in microvascular myographs for isometric tension recordings as described previously[26] and maintained in oxygenated PSS (5% CO_2 in 95% O_2; 1.6 mmol/L $CaCl_2$) at 37°C. During equilibration of the vessels, tension (T) corresponding to a pressure (P) of 70 mm Hg according to the equation $P = T2\pi U^{-1}$ (U = inner circumference) was adjusted. In all experiments cyclooxygenase-mediated relaxation was blocked with cyclooxygenase inhibitor diclofenac (0.1 mmol/L; Sigma, Taufkirchen, Germany). The vessel rings were contracted with cumulatively increasing concentrations of phenylephrine (PE). Relaxation was measured by increasing concentrations of acetylcholine (ACh) or sodium nitroprusside (SNP) in PE-precontracted (10 μmol/L) vessels. Relaxing effects of ACh were studied in the absence and presence of NO synthase inhibitor nitro-L-arginine-methylester (L-NAME; 30 μmol/L), BK_{Ca} inhibitor paxilline (1 μmol/L), cytochrome P450 inhibitor N,N-diethylaminoethyl-2,2-diphenylvalerate (proadifen; 50 μmol/L), and epoxygenase inhibitor 6-(2-propargyloxyphenyl)hexanoic acid (PPOH; 30 μmol/L). The effects of ACh or SNP are expressed in percent of the response to PE (=100%).

2.6 Isolation of vascular cells

Mesenteric arteries were stored in low Ca^{2+}-containing PSS (0.16 mmol/L Ca^{2+}) at 4°C. Enzymatic dissociation was carried out in two steps. The first solution (1 mL PPS) contained 0.7 mg papain; 1.5 mg dithioerythritol (Roth, Karlsruhe, Germany), bubbled with O_2, 20 min, 37°C. The second solution (1 mL PPS) contained 1.2 mg collagenase type F, 1.5 mg trypsin inhibitor, 0.5 mg elastase (Serva, Heidelberg, Germany), and 1.0 mg bovine albumin fraction V (Serva, Heidelberg, Germany) gassed with O_2, 12 min, 37°C. Single VSMCs and ECs were obtained by trituration in PSS.

2.7 Electrophysiological experiments

Potassium outward current through the BK_{Ca} channels ($I_{BK,Ca}$) was measured using a HEKA-EPC8 amplifier (HEKA Elektronik, Lambrecht, Germany) in voltage-clamp mode. Bath superfusion buffer (mmol/L): NaCl 127.0; KCl 5.9; $CaCl_2$ 2.4; $MgCl_2$ 1.2; glucose 11.0; HEPES 10.0, pH 7.4. Pipette solution (mmol/L): KCl 134.0; NaCl 6.0; $MgCl_2$ 1.2; $CaCl_2$ 4.2, EGTA 5.0; glucose 11.0; Mg-ATP 3.0; HEPES 10.0; pH 7.4. Pipette tip resistance was 3.0–4.0 MΩ. Experiments were carried out at 21°C. Effects of the following compounds were studied: 1,3-Dihydro-1-[2-hydroxy-5- (trifluoromethyl)phenyl]-5-(trifluoromethyl)-2H-benzimidazol-2-one (NS1619; 30 μmol/L), paxilline (1 μmol/L), H_2O_2 (1 μmol/L), 11,12-Epoxy-(5Z,8Z,14Z)-

eicosatrienoic acid (11,12-EET; 300 nmol/L), and 14(R),15(S)-Epoxy-(5Z,8Z,11Z)-eicosatrienoic acid (14,15-EET; 300 nmol/L). The cell capacity was used for data normalization. Unless stated otherwise, all substances were purchased from Sigma, Taufkirchen, Germany.

2.8 RNA isolation and real-time PCR

Total RNA of mesenteric arteries was isolated using the EZNA Total RNA-Kit (Peqlab, Erlangen, Germany). For quantification of mRNA expression real-time PCR was performed using the QuantiTect SYBR Green RT-PCR kit (Qiagen, Hilden, Germany) in a thermal cycler (Corbett Research, Mortlake, Australia). Primers: bovine LOX-1 (sense: 5′-CCAGGAGAACTGCTTGTCTT-3′, antisense: 5′-GTGC TCTCAATAGATTCGCC-3′), eNOS (sense: 5′-TTCCGGCTGCCACCTGATC CTAA-3′, antisense: 5′-AACATATGTCCTTGCTCAAGGCA-3′), KCNMA (sense: 5′-CGATAAGCTGTGGTTCTGGC-3′, antisense: 5′-AAGAAGACC ATGAAGAGGCGTC-3′), and KCNMB1 (sense: 5′-AGAAGCGCGGAGAGA CACGA-3′, antisense: 5′-CAGCTCTTCCTGGTCCTTGATA-3′). Quantification by one-step RT real-time PCR included 50°C for 30 min, 95°C for 15 min, subsequent cycles of 94°C for 30 s, 60°C for 30 s, and finally 72°C for 50 s. Internal RNA standards were produced as previously described.[27]

2.9 Data analysis and statistics

Potency of agonists was determined as $-\log EC_{50}$ [mmol/L]. All results are expressed as mean ± standard error of the mean (SEM). Unless stated otherwise, number of experiments is given as arteries or cells from *n* mice. Student's *t*-test (unpaired) was used for statistical analysis, differences with $P < 0.05$ were considered significant. Multiple comparisons were done by one-way ANOVA followed by Bonferroni *post hoc* test.

3. Results

3.1 Body weight and serum lipids

Table 1 summarizes body weight and serum lipid concentrations in the four investigated animal groups. LOX-1 mice on normal diet were heavier than WT mice, but did not differ in serum lipid concentrations. FD significantly increased body weight in WT and in transgenic mice when compared with control diet. While serum concentrations of triglycerides were not different between the groups, free cholesterol, LDL, and HDL concentrations were significantly higher in WT and LOX-1 animals on high-fat compared with standard diet. The ratio of LDL:HDL increased during FD from 1:7 to 1:4 in WT mice and from 1:8 to 1:4 in LOX-1 mice. Although FD caused significantly larger weight gain in LOX-1 mice compared with WT animals, no statistically significant differences were found in serum lipid parameters.

3.2 Vascular function

Basal tone of mesenteric arteries was unaffected by FD (compare pre-PE control values in Supplementary material online, *Figure S2A*).

Endothelium-dependent and -independent relaxation in mesenteric arteries was studied in PE-preconstricted vessels. In order to determine the optimum concentration for precontraction, we measured contractile responses to cumulatively increasing PE concentrations. All vessels contracted in the same concentration range [average $-\log EC_{50}$ (mol/L) values between 5.5 and 5.7]. However, the maximum contractile response to PE was significantly lower in the LOX-1 + FD mice than in the other groups (LOX-1 + FD: 1.4 ± 0.1 mN/mm vs. WT: 1.7 ± 0.3 mN/mm; WT + FD: 1.8 ± 0.1 mN/mm; LOX-1: 2.0 ± 0.2 mN/mm; $P < 0.05$; see Supplementary material online, *Figure S2A*). Subsequently, arteries in all further experiments were precontracted with 10 μmol/L PE. Values of maximum contraction produced by 80 mmol/L KCl were similar in the four groups (see Supplementary material online, *Figure S2C*). Absolute values of the constriction induced by 100 μM PE and 80 mmol/L KCl in WT and LOX-1 mice are consistent with previously published results.[28,29]

Endothelium-dependent relaxation was studied by exposing the arteries to increasing concentrations of ACh. The resulting concentration–response curves (CRC; *Figure 1A* and *B*) revealed incomplete relaxation by ACh with significantly reduced relaxation in LOX-1 + FD animals (maximum relaxation, Eff_{max}: 67.8 ± 3.4%; $P < 0.01$) compared with WT, WT + FD, and LOX-1 (Eff_{max}: 89.3 ± 5.0%; 88.1 ± 4.7%; and 88.1 ± 3.6%; *Figure 1C*). Interestingly, potencies were similar in all groups [average $-\log EC_{50}$ (mol/L) values between 6.8 and 7.0]. The relaxing response to ACh was reduced in the presence of the NO synthase (NOS) blocker L-NAME (30 μmol/L). Relaxation was reduced in WT (42.2 ± 5.6%), WT + FD (39.1 ± 7.5%), and LOX-1 mice (40.1 ± 6.1%), but not in LOX-1 + FD arteries (5.5 ± 5.2%). These results suggest that NO-mediated relaxation was significantly impaired in LOX-1 + FD animals compared with the other groups (*Figure 1D*). In addition the basal tone during L-NAME incubation increased only in arteries from WT, WT + FD, and LOX-1 animals, but not in LOX-1 + FD mice. In the presence of L-NAME, the

Table 1 Characterization of body weight and serum parameters

	WT		LOX-1	
	Control	+FD	Control	+FD
Body weight, g (*n*)	27.3 ± 0.8 (8)	33.1 ± 1.1** (22)	30.7 ± 1.2# (7)	36.3 ± 1.5*# (18)
Triglycerides, mmol/L (*n*)	1.1 ± 0.1 (7)	1.3 ± 0.1 (24)	1.1 ± 0.03 (7)	1.3 ± 0.1 (18)
Cholesterol, mmol/L, (*n*)	3.4 ± 0.2 (7)	5.6 ± 0.3*** (24)	3.3 ± 0.1 (7)	5.9 ± 0.3*** (18)
HDL, mmol/L, (*n*)	2.7 ± 0.2 (7)	4.5 ± 0.2*** (24)	2.4 ± 0.1 (7)	4.8 ± 0.3*** (18)
LDL, mmol/L, (*n*)	0.3 ± 0.1 (7)	1.1 ± 0.1*** (24)	0.3 ± 0.04 (7)	1.1 ± 0.1*** (18)

*$P < 0.05$.
**$P < 0.01$.
***$P < 0.001$ standard diet vs. high-fat diet.
#$P < 0.05$ WT vs. LOX-1.

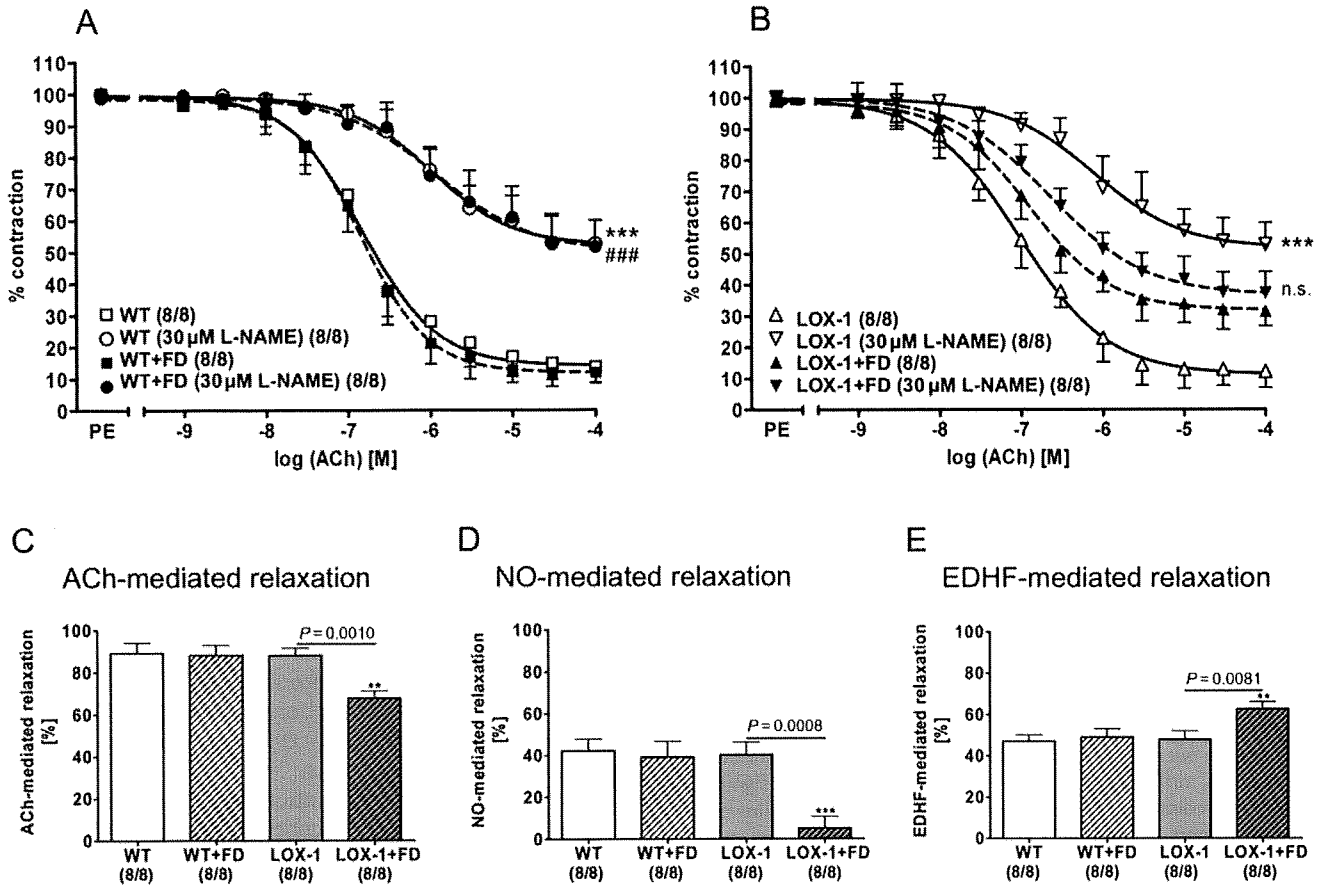

Figure 1 Effects of acetylcholine in mesenteric arteries of WT and LOX-1 mice. (*A*) Concentration–response curves (CRCs) for acetylcholine in arteries of WT mice on standard and high-fat diet without and with L-NAME. (*B*) CRCs for acetylcholine in arteries of LOX-1 mice on standard and high-fat diet without and with L-NAME. Maximum effects of acetylcholine-induced (*C*), NO-mediated relaxation (*D*), and EDHF-mediated relaxations (*E*) of arteries from WT and LOX-1 mice on standard and high-fat diet. $^{***}P < 0.001$ control vs. L-NAME of mice on standard diet. $^{\#\#\#}P < 0.001$ control vs. L-NAME of mice on high-fat diet.

differences in PE-induced contractions between LOX-1 + FD mice and the other three animal groups persisted (see Supplementary material online, *Figure S2D* and *E*).

ACh-induced relaxation in the presence of NOS and cyclooxygenase inhibitors is mediated by EDHF. The EDHF-mediated fraction of relaxation was largest in LOX-1 + FD mice (62.6 ± 3.5%) compared with the other groups (WT: 47.0 ± 3.2%; WT + FD: 49.0 ± 4.0%; LOX-1: 48.0 ± 4.1%; $P < 0.01$; *Figure 1E*). Cytochrome P450 enzymes are a substantial source of EDHF, they are responsible for the transformation of arachidonic acid into epoxyeicosatrienoic acids (EETs).[30,31] The role of these enzymes in ACh-induced relaxation was examined with proadifen, a non-specific blocker of cytochrome P450 isoenzymes and PPOH, a specific epoxygenase blocker. Proadifen and PPOH did not significantly block EDHF-mediated relaxation in WT, WT + FD, and LOX-1 animals, but significantly reduced EDHF-mediated relaxation in LOX-1 + FD (proadifen 41.3 ± 6.3%; $P < 0.01$ and PPOH 28.7 ± 6.3%; $P < 0.05$; *Figure 2A–D*). Both compounds changed the efficacy of ACh to a larger extent in LOX-1 + FD mice compared with the other groups, leading to similar levels of relaxation in all four groups.

The contribution of BK_{Ca} channels as potential EDHF targets in LOX-1 + FD was tested by blocking the channels with the specific BK_{Ca} blocker paxilline (*Figure 2E–H*). Paxilline reduced ACh-mediated relaxation only in LOX-1 + FD (18.42 ± 3.6%; $P = 0.0026$), but not in WT (0.1 ± 3.3%), WT + FD (1.6 ± 2.5%), and LOX-1 animals (1.1 ± 3.9%), indicating that BK_{Ca} channels were activated only in LOX-1 + FD mice. Even in the presence of L-NAME, paxilline was able to inhibit relaxation, indicating a BK_{Ca} channel involvement in the EDHF-mediated relaxation. During paxilline incubation the basal tone significantly elevated in arteries from LOX-1 + FD mice compared with WT, WT + FD, and LOX-1 animals (see Supplementary material online, *Figure S2F*). PE-induced contraction in the presence of paxilline was similar in all four groups (see Supplementary material online, *Figure S2G*).

Endothelium-independent relaxation was measured using the NO-donor SNP. Potency and efficacy of SNP were calculated from cumulative CRCs (see Supplementary material online, *Figure S3*) and were found to be similar in all groups [$-\log EC_{50}$ (mol/L): WT: 7.4 ± 0.2; WT + FD: 7.4 ± 0.1; LOX-1: 7.2 ± 0.2; LOX-1 + FD: 7.2 ± 0.1; Eff_{max}: WT: 0.6 ± 2.5%; WT + FD: 1.2 ± 2.6%; LOX-1: 2.8 ± 2.9%; LOX-1 + FD 1.7 ± 0.5%].

3.3 Reactive oxygen species

Since vascular dysfunction is associated with increased ROS production, we have examined the formation of ROS by chemiluminescence in mesenteric arteries. ROS formation was increased in WT + FD and LOX-1 + FD, and the increase

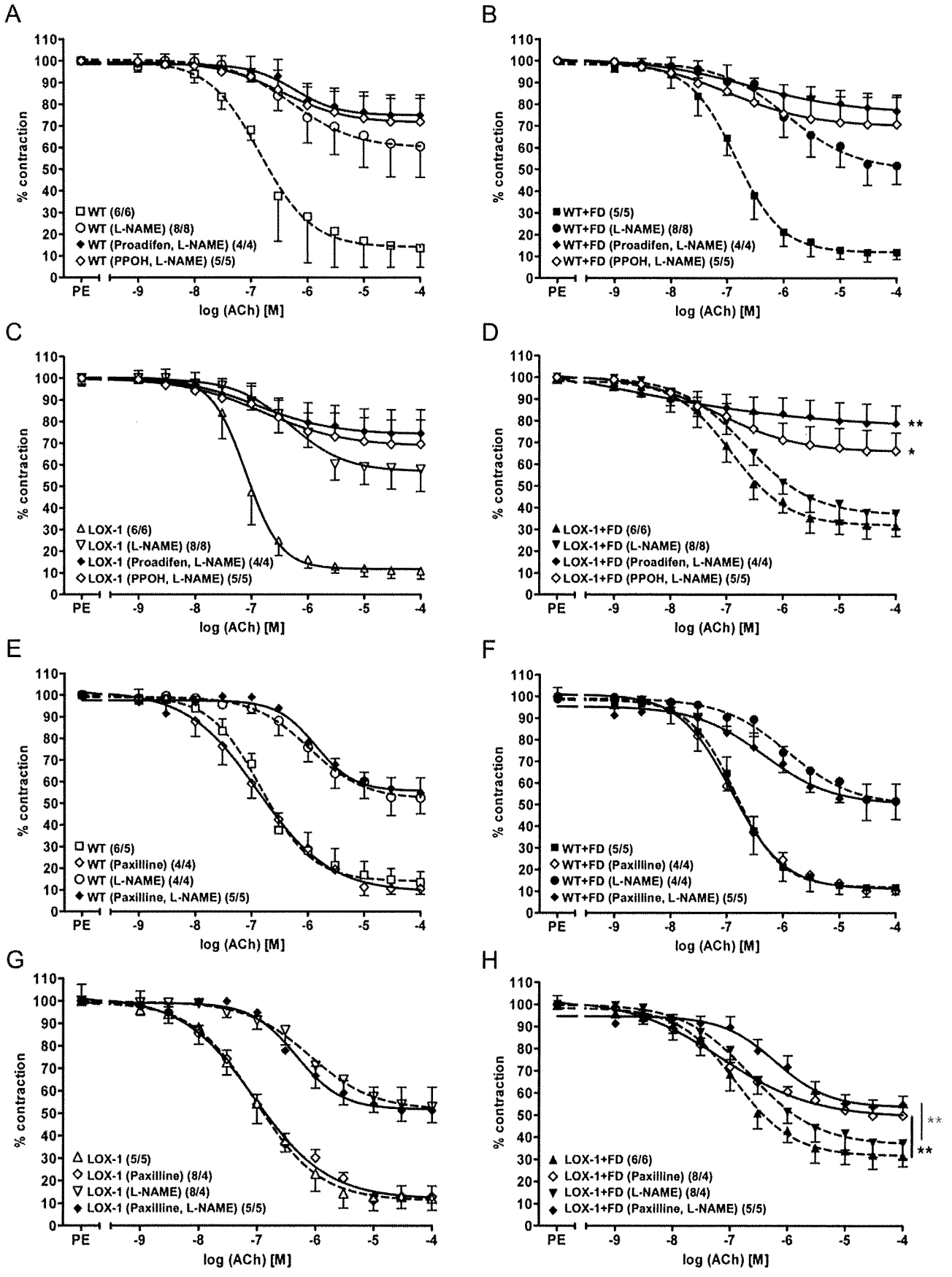

Figure 2 Concentration–response curve for acetylcholine in the presence of BK_{Ca} channel blocker and cytochrome P450 blocker in mesenteric arteries of WT and LOX-1 mice. Concentration–response curves (CRCs) for acetylcholine in the presence of cytochrome P450 blocker proadifen and epoxygenase blocker PPOH in arteries of (*A*) WT; (*B*) WT + FD; (*C*) LOX-1; (*D*) LOX-1 + FD in combination with L-NAME. CRCs for acetylcholine in the presence of BK_{Ca}-channel blocker paxilline on the arteries of (*E*) WT; (*F*) WT + FD; (*G*) LOX-1, and (*H*) LOX-1 + FD and in the presence and absence of L-NAME. *$P < 0.05$ proadifen + L-NAME vs. L-NAME; **$P < 0.01$ PPOH + L-NAME vs. L-NAME.