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G. 知的財産権の取得状況

1．特許取得

なし

2．実用新案登録

なし

3．その他

厚生労働省科学研究費補助金（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

# 2型糖尿病患者のQOL、血管合併症及び長期予後改善のための前向き研究（JDCStudy）
## 糖尿病の治療に関する研究－JDCSの問題点と提案

分担研究者　森　保道　虎の門病院内分泌代謝科

研究要旨
JDCS 研究は日本人2型糖尿病についての大規模かつ長期間にわたる臨床研究である。本研究を遂行する上での問題点についてその要因を検討した。

A. 研究目的
JDCS の問題点を挙げ、その解決を目的とした。

B. 研究方法
本研究実施上の現状と問題点を把握し、対策を検討した。

C. 研究結果と考察
① 当施設の登録患者における現状と問題点
症例調査が指定年度施行制へ移行し、本年度は調査票の作成年次であった。対象症例から調査への継続的な協力が得られ、下記3カ年の定期的検査の経過につき報告書を作成した。
（ⅰ）平成17年4月～平成18年3月分（ⅱ）平成18年4月～平成19年3月分
（ⅲ）平成19年4月～平成20年3月分
検査の円滑な施行については、診療録への事務局からのシールの添付は非常に有効であり、さらに平成18年より電子オーダリングシステムによる予約・検査管理が可能となり、JDCS 参加者の受診予定を事前に確認し、受診前日に担当医に検討事項の一覧の附箋を渡した対応が奏功した。また、長期予後に関する調査の観点から、非通院症例に対しても転院先ないしは直接の連絡によって可能な限り健康状態の確認を施行した。調査対象症例の高齢化に伴い悪性疾患を合併する症例が多く認められ、死亡例も認められた。本研究の意義は広く理解されており、ほとんどの医療機関から情報収集への協力が得られた。

② 長期予後解析について
2008 年に英国で施行された大規模臨床試験であるＵＫＰＤＳの長期観察研究が報告され、適切な血糖管理に向けた早期からの介入が2型糖尿病の大血管合併症発症を抑制し、死亡率の低下に寄与することが提示された。ＵＫＰＤＳは1970年代から開始され、30年余の調査が今日糖尿病治療の根幹をなすエビデンスを形成している。
糖尿病の病態は複雑であり長期予後を規定する因子は多岐にわたる。今年度ＪＤＣＳは過去3年間にわたる疾患の罹患状態を集積することにより、今日の2型糖尿病の慢性合併症に関する広範なデータベースが形成され、2型糖尿病の長期予後因子を解析する貴重な資料となることが期待される。

D. 結論
今年度の症例調査により平成20年3月時までの臨床成績が集積された。大血管合併症への関心が高まる中 JDCS は日本人2型糖尿病の最新のエビデンスを提供する臨床研究として位置づけられている。糖尿病慢性合併症の発現状況や死亡率などの貴重な成果は、日本人2型糖尿病の医療対策を構築するうえで最も信頼される EBM であると考えられる。

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

2型糖尿病患者のQOL、血管合併症及び長期予後改善のための前向き研究（JDCStudy）

# JDCStudyの問題点とその解決

分担研究者　沖田考平　大阪大学大学院医学系研究科内分泌代謝内科

研究要旨

本研究は日本人２型糖尿病における血管合併症の予防、進展抑制を目的とした大規模臨床研究である。研究を遂行する上での問題点を把握し、その要因について検討した。

A. 研究目的

本研究は、我が国における糖尿病患者の細小および大血管合併症の発症状況を把握し、その予防・進展抑制をはかるための介入効果を検討した我が国独自の大規模臨床研究として成果を上げており、国際的にも高い評価を得ている。そこでさらに質の高い研究とするため、現在の問題点とその対策について検討した。

B. 研究方法

本研究を実施するにあたり、現状と当施設における遂行上の問題点を把握し、その対策について検討を行った。

C. 研究結果と考察

(1)　登録患者における問題点

本年度において転院された症例、悪性腫瘍のため死亡された症例が認められた。本研究は長期にわたっており、登録患者は転居などの理由により他院へ通院する、本人・家族等の問題により通院継続が困難になるなどの理由で、今後も脱落したり追跡が困難になる症例が出現する可能性がある。本研究の目的を遂行するためには、脱落症例を出来るだけ少なくすることが必要である。通院継続の為にはデータをフィードバックするなど患者のモチベーションを上げる努力を行う必要がある。

(2)　医療サイドにおける問題点

本研究は長期におよんでいるため主治医の交代も多い。主治医交代時には十分な申し送りを行うとともに、登録証のシールを有効に活用し、確実に治療・検査を行うことが必要である。また、患者が転院した場合は、転院先と連携し追跡を続ける努力が必要であるが、医療機関によっては糖尿病専門医が退職し、研究継続に支障をきたしている病院も認められる。さらに高い質の研究を続けるためには、事務局と医療機関、主治医および患者の間で十分な連携をとり確実に追跡していく努力が必要がある。

D. 結論

本研究は日本人２型糖尿病患者において多くの診療エビデンスを生み出してきた。今後は、細小および大血管合併症の発症状況に加え死亡率の検討なども必要であると考えられるが、その場合高齢などの理由で通院困難となった例も確実に追跡調査する必要がある。また担当医師の異動も多く、当初の目的を遂行する上で支障をきたすこともあるが、患者、主治医および事務局との十分なコミュニケーションをはかり、登録患者を最後まで追跡していく努力をすることが必要である。本研究により日本人2型糖尿病患者の血管障害の発症や増悪に関与する因子が明らかになるものとして期待される。

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
2型糖尿病患者のQOL、血管合併症及び長期予後改善のための前向き研究（JDCS）

# JDCSの課題と今後への提案

研究分担者　水流添　覚　熊本大学医学部附属病院　代謝・内分泌内科

研究要旨
研究を遂行する上で問題となる臨床現場における課題について、当施設においてその要因を解析・検討し、また今後の研究遂行について考察した。

A．研究目的

本研究は日本人糖尿病患者独自の特徴を発見するなど質の高い大規模臨床研究として国際的にも評価されている。研究の質の高さを保つため、当施設における本研究遂行の現状と問題点を整理することで、本研究の課題とその対策を検討せんとした。

B．研究方法

当施設における本研究実施上の現状と問題点を把握し、対策と今後の展望を検討した。

C．研究結果

①調査項目・隔年毎のデーター報告

検査項目を確実に網羅するためにJDCS事務局からのシールを利用して情報収集を行った。ただし、診療録の更新などの理由で有効利用できない例があるため、予備シールがあれば、より良いと考えられた。調査項目については、妥当である。

②登録患者脱落について

当施設における研究登録患者は開始時の割り付け患者45名であったが、登録患者は次第に高齢化しており、脱落例も多い。2009年1月1日時点での患者平均年齢73.1歳（61才ー80才）、75歳以上の後期高齢者6人含まれる。

当施設におけるこれまでの調査脱落の主な理由は、患者死亡、転居、来院中止等であった。

D 考察

調査項目の縮減は、登録患者、医療者ともに負担が減少している。隔年毎のデーター報告はデーターの抜けの懸念があったが、シール等により必要項目を周知した結果、情報抜けは少なかったと考える。

またこれまでの調査脱落の主な理由は、経済的理由以外での登録中止が多かったが、高齢者の医療費自己負担率の増に伴い経済的理由による通院中断などが懸念される。特に登録患者の糖尿病罹病期間が長期化しており、インスリン使用率（当科では現在約40%）が高くなってきている。

担当医師の異動に伴う引継ぎの不手際が見られる場合もあるが、JDCS事務局からのシールはその防止に有効であった。ただ外来担当医師の多くはJDCS研究途中から研究に参加しており、研究の意義や実際について、十分に理解していない面も見受けられ、随時担当医側の教育も必要である。

一方、新たな臨床検査法がいくつか広く臨床応用されてきているが、当科においても糖尿病患者における冠動脈疾患のスクリーニングとしてMDCTを用いた解析を行っており、有用性を確認している。このような新検査法でのスクリーニング結果をソフトイベントに組み入れるか考慮すべきかと思われた。

E．結論

・調査項目の縮減・隔年毎のデーター報告、は研究の遂行に利する。
・JDCS事務局からのシールは有効に機能しており毎年の配布が望まれる。
・高齢化および医療制度の変化を考慮しつつ研究を遂行する必要がある。

G．研究発表

学会発表

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分担研究報告書

# 2型糖尿病患者のQOL、血管合併症および長期予後改善のための前向き研究

分担研究者　松久宗英　大阪大学大学院内分泌・代謝内科　助教

研究要旨
2型糖尿病とその血管合併症（網膜症、腎症、神経障害および大血管合併症）に関する約2000人を対象とした前向き観察試験である。生活指導等を強化した介入群と非介入群において、血管合併症の発症頻度とその危険因子を明らかとし、糖尿病合併症の発症および進展阻止を可能とする治療の実現へ貢献する。

## A. 研究目的

本研究は、わが国の2型糖尿病患者における血管合併症の発症・進展にかかわる因子を抽出し、その抑制のためのエビデンスを確立し、患者の生命予後とQOLの改善へ貢献することを目的とする。この研究の中で、我々は特に心血管疾患を中心とした大血管合併症の評価を担当する。

## B. 研究方法

「糖尿病における血管合併症の発症予防と進展抑制に関する研究（JDCS: Japan Diabetes Complication Study）」において、約2000例の対象に生活習慣指導を中心とした強化療法による介入群と、非介入群における前向き観察研究として、その病態と糖尿病血管合併症の発症頻度および危険因子を経年的に評価解析する。

## C. 研究結果

現在まで12年間の観察が行われ、800例以上の症例で8年次のdata固定が行われた。糖尿病大血管合併症の発症頻度は、虚血性心疾患および脳梗塞ともに経年的に増加し、近年では心筋梗塞の発症頻度が脳梗塞よりも高率となり、日本の疾病状態が欧米型に近づきつつあることが明らかとなった。また、危険因子として高血糖（HbA1c）、LDL-コレステロール、中性脂肪、収縮期血圧などの古典的危険因子が、本研究でも大血管合併症と関連することが明かとなった。また、脂質関連解析において、Non-HDLコレステロールの重要性も明らかにされた。しかしながら、生活習慣への介入が持つ意義は明らかにできなかった。

## D. 考察

本研究における12年に及ぶ長期の観察により、日本人の2型糖尿病患者における細小血管から大血管の合併症の危険因子が解明されてきた。
近年のACORD, ADVANCEなど海外での大規模研究では、高血糖に対する積極的な介入が、大血管障害を抑制できないことが報告される中、高血糖が本邦の心血管疾患リスクであることが明確になったことは特筆すべき成果である。糖尿病合併症の研究において、民族性や生活環境の特色を踏まえたものになるためには、本研究のような長期の多数例のものが必要不可欠である。したがって本研究は、稀少なコホート研究であり、今後も長期に継続することは意義深い。さらに、他研究では殆ど検討されていない日本人の食生活習慣に立脚した解析は今後の成果が期待される。

今回生活習慣への介入が糖尿病血管合併症の発症進展に抑制効果を示さなかったが、これは必ずしも生活介入が意味のないことを示すものではなく、既にある程度厳格な生活介入が行われていたため、介入群と非介入群での差が明確とならなかったと考える。また、近年Legacy Effectとして知られるようになった、長期間に及ぶ介入効果に関しても、今後の継続的検討が待たれる。

現在までの2型糖尿病患者における心血管疾患の危険因子の解析からも、その発症・進展の阻止に向けたアプローチが現実のものとなってきた。本研究で得られたエビデンスを今後広く啓発していくとともに、糖尿病大血管障害の進展阻止のための治療指針を作成し、臨床現場に着実に活かしていく必要がある。

## E. 結論

生活の変化とともに、わが国の糖尿病合併症も欧米型へ変化を遂げており、今後糖尿病血管合併症を予防するためには、高血糖の是正とともに複数の関連危険因子を厳格に管理していくことが重要であると考えられた。

## F. 研究発表　特になし

知的財産権の出願・登録状況　特になし

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
2型糖尿病患者のQOL、血管合併症及び長期予後改善のための前向き研究（JDCS）

# JDCSの課題と今後への提案

分担研究者　林　登志雄　名古屋大学医学部附属病院老年医学

研究要旨　JDCS研究は日本人2型糖尿病についての大規模かつ長期間にわたる臨床研究である。本研究を遂行する上での問題点についてその要因を検討し、今後の研究遂行について考察した。

## A. 研究目的

本研究は日本人2型糖尿病患者を対象に、病態及びその各合併症の発症頻度、要因、危険因子等につき、本邦独自の特徴を発見、報告してきた。本邦の大規模臨床研究を代表する質の高い研究として国際的にも評価されている。１０年余に及ぶ世界的にも類い稀な長期研究である本研究が今後もその質の高さを保つため、本研究遂行の現状と問題点を整理することで、その課題と対策を検討せんとした。

## B．研究方法

本研究登録患者の平均年齢が第9年次には男女とも67歳をこえ、その後の調査での回収率も対象患者の治療施設が変わったために低下しているとの報告が増えている。分担研究者が属する老年医学の立場から本研究実施上の現状と問題点を把握し、対策と今後の展望を検討した。

## C．研究結果と考察

①8年次データ固定・調査項目の縮減・隔年毎のデーター報告について

9年次より開始された調査項目の縮減は、医療現場において診療の負担が減少し、登録患者ならびに担当医側の双方に良好に受け入れられていると感じた。これだけの長期研究は他に類をみず、どうしても登録患者の脱落、登録項目調査の逸脱等が生じると考えられる。事務局主導で登録時から第8年次にまでのデータの固定が行われた事は論文発表のみならず、今後の研究継続上も有意義だと考えられた。隔年毎のデーター報告も担当医が交代されていく中，負担軽減となっている。但し、データーの抜けの懸念がある。９、１０年次の現状でのデータ回収率は、対象患者が登録時の医療機関に継続受診できなくなっている現状を反映していると考える。

②登録患者における現状と課題

登録患者が高齢化しており、通院困難などの理由により他医院や他科との連携が今後は重要になろう。一部の患者は後期高齢者になっており主治医制度の下で、診療所と連携する必要も出てくると考える。

③登録患者の高齢化に起因する課題

本研究の主題である血管合併症においては登録患者の高齢化により当初と危険因子が異なってくる可能性がある。また女性の発症が増加すると思われる年代である。脂質、血圧等を含め年代別縦断成績での解析等が望まれる。我々が別に開始しているコホート群でも同様の結果が示されつつあり、縦断研究として本邦最大の規模を誇る当該研究でもそういった発表がなされれば社会的インパクトも大きいと考える。

一般的問題として、1）高齢者を個別には何歳まで観察するか、2）高齢者で増加する脳血管合併症や認知症にどう取り組むか、心不全を虚血性あるいはその他の原因にどのように分類するか。3）ADL障害(寝たきり)も含め、除外基準を作るか等を評価する必要があると考える。また，報告されているようにしまい研究と位置づけられるJEDIT等、本邦で行われている高齢者糖尿病研究とのデータの比較等も可能になってきたと考える。

④今後への提言

本研究が開始され既に10年経過しており、既登録患者の減少・脱落が懸念される。日本人における糖尿病血管合併症の発症進展に関する新しい解析のため、新規患者を登録して、新たな観察対象群を設定する試みも必要かと考えられる。また最近の検査成績，治療成績の組み入れ，統計への反映も検討できると意義は大きいと考える。

私どもが開始しているコホート群も平均

年齢が近く有用かもしれない。

D．結論

患者高齢化、医師の異動によるデータ脱落を最小限とすべく、周辺医療機関との連携等により長期追跡をおこなうことが重要と考えられる。

大血管合併症への関心が高まる中 JDCS を通じ日本人２型糖尿病の最新のエビデンスが提供され、成果は日本人２型糖尿病の医療対策を構築する上で最も信頼される EBM である。高齢社会日本をも代表する成績に育つと期待される。

現在の登録患者のみならず、年齢を一致させた新規の患者登録も考慮する必要があるかもしれない。

F．健康危険情報

なし

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H.知的財産権の出願・登録状況

なし

別紙4

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Biochemical and Biophysical Research Communications xxx (2009) xxx–xxx

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

# Angptl 4 deficiency improves lipid metabolism, suppresses foam cell formation and protects against atherosclerosis

Hironori Adachi [a], Yukio Fujiwara [b], Tatsuya Kondo [a], Takeshi Nishikawa [a], Rei Ogawa [c], Takeshi Matsumura [a], Norio Ishii [a], Ryoji Nagai [d], Keishi Miyata [e], Mitsuhisa Tabata [e], Hiroyuki Motoshima [a], Noboru Furukawa [a], Kaku Tsuruzoe [a], Junji Kawashima [a], Motohiro Takeya [b], Shizuya Yamashita [f], Gou Young Koh [g], Andras Nagy [h], Toshio Suda [i], Yuichi Oike [e,*], Eiichi Araki [a,*]

[a] Department of Metabolic Medicine, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
[b] Department of Cell Pathology, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
[c] Department of Environmental & Symbiotic Sciences, Prefectural University of Kumamoto, 3-1-100 Tsukide, Kumamoto 862-8502, Japan
[d] Department of Medical Biochemistry, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
[e] Department of Molecular Genetics, Faculty of Medical and Pharmaceutical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan
[f] Department of Cardiovascular Medicine, Osaka University Graduate School of Medicine, 2-2, Yamadaoka, Suita, Osaka 565-0871, Japan
[g] Biomedical Center, Department of Biological Sciences, Korea Advanced Institute of Science and Technology, Daejeon, 305-701, Republic of Korea
[h] Department of Medical Genetics and Microbiology, University of Toronto, Toronto, ON, Canada M5S 1A8
[i] Department of Cell Differentiation, The Sakaguchi Laboratory, School of Medicine, Keio University, Tokyo 160-8582, Japan

ARTICLE INFO

*Article history:*
Received 25 November 2008
Available online xxxx

*Keywords:*
Angptl 4
Knockout mice
Apolipoprotein E
Atherosclerosis
Lipid metabolism
Lipoprotein lipase
Postprandial hypertriglyceridemia
Macrophage
Foam cells
Foam cell formation
Oxidized low-density lipoprotein
Lipoprotein lipase

ABSTRACT

Angiopoietin-like protein family 4 (Angptl 4) has been shown to regulate lipoprotein metabolism through the inhibition of lipoprotein lipase (LPL). We generated ApoE$^{-/-}$Angptl 4$^{-/-}$ mice to study the effect of Angptl 4 deficiency on lipid metabolism and atherosclerosis. Fasting and postolive oil-loaded triglyceride (TG) levels were largely decreased in ApoE$^{-/-}$Angptl 4$^{-/-}$ mice compared with and ApoE$^{-/-}$Angptl 4$^{+/+}$ mice. There was a significant (75 ± 12%) reduction in atherosclerotic lesion size in ApoE$^{-/-}$Angptl 4$^{-/-}$ mice compared with ApoE$^{-/-}$ Angptl 4$^{+/+}$ mice. Peritoneal macrophages, isolated from Angptl 4$^{-/-}$ mice to investigate the foam cell formation, showed a significant decrease in newly synthesized cholesteryl ester (CE) accumulation induced by acetyl low-density lipoprotein (acLDL) compared with those from Angptl 4$^{+/+}$ mice. Thus, genetic knockout of Angptl 4 protects ApoE$^{-/-}$ mice against development and progression of atherosclerosis and strongly suppresses the ability of the macrophages to become foam cells *in vitro*.



Dyslipidemia is one of the major risk factors for cardiovascular disease. It is usually assessed by the fasted levels of low-density lipoprotein (LDL), triglyceride (TG), and high-density lipoprotein (HDL) [1]. In addition to the fasting dyslipidemia, recent studies have suggested that postprandial hypertriglyceridemia accelerates arteriosclerosis, such as myocardial infarction [2]. Lipoprotein lipase (LPL) plays crucial roles in lipid metabolism and transport by catalyzing the hydrolysis of TG-rich lipoproteins such as chylomicron and very-low-density lipoprotein (VLDL). For example, overexpression of LPL in LDL receptor knockout mice strongly suppresses postprandial hypertriglyceridemia [3,4]. In general, LPL is viewed as an anti-atherogenic enzyme because of its action on circulating fasting and postprandial lipoproteins [3–5].

Recently, a family of proteins, structurally similar to the angiogenic regulating factors angiopoietins, was identified and designated "angiopoietin-like proteins" (Angptls). Encoded by seven genes, Angptls 1 to 7 all possess an N-terminal coiled-coil domain and a C-terminal fibrinogen-like domain, both of which are characteristic of angiopoietins. Angptls 3 and 4, and Angptl 6/angiopoietin-related growth factor also appear to directly regulate lipid, glucose and energy metabolism independently of angiogenic effects [6].

Angptl 4 has been shown to regulate lipid metabolism mainly by inhibiting LPL activity [7–10]. More recently, the mechanism of LPL inhibition by Angptl 4 has been unveiled. The N-terminal

* Corresponding authors. Fax: +81 96 373 5145 (Y. Oike), +81 96 366 8397 (E. Araki).
*E-mail addresses:* oike@gpo.kumamoto-u.ac.jp (Y. Oike), earaki@gpo.kumamoto-u.ac.jp (E. Araki).

0006-291X/$ - see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.bbrc.2008.12.018

Please cite this article in press as: H. Adachi et al., Angptl 4 deficiency improves lipid metabolism, suppresses foam cell ..., Biochem. Biophys. Res. Commun. (2009), doi:10.1016/j.bbrc.2008.12.018

coiled-coil domain of Angptl 4 binds transiently to LPL and this interaction results in the conversion of the enzyme from catalytically active dimers to an inactive form [11]. Consistent with these findings, Angptl $4^{-/-}$ mice exhibited 65–90% lower fasting TG levels and slightly lower total cholesterol (T-CHO) levels, in addition to lower circulating VLDL and an increase in LPL activity [8–10].

As discussed above, the effect of Angptl 4 on lipid metabolism has gradually become clear; however, it is still unknown whether Angptl 4 directly affects the development and progression of atherosclerosis. In addition, no study has previously examined the role of Angptl 4 in the formation of foam cells from macrophages.

In this study, we used the atherosclerosis-prone $ApoE^{-/-}$ mouse to show that genetic knockout of Angptl 4 is sufficient to protect these mice against development and progression of atherosclerosis. We also show that the absence of Angptl 4 strongly suppresses the ability of the macrophages to become foam cells *in vitro*.

## Materials and methods

*Animals.* Angptl $4^{+/-}$ mice were generated by Koh and Nagy as previously described [8]. Angptl $4^{+/+}$ and Angptl $4^{-/-}$ mice littermates were obtained from crosses of Angptl $4^{+/-}$ mice with C57BL/6J background, and Angptl $4^{-/-}$ mice were crossed with $ApoE^{-/-}$ mice (B6.129P2-Apoetm1Unc; The Jackson Laboratory) to generate $ApoE^{+/-}$Angptl $4^{+/-}$ mice. These mice were used as breeding pairs to generate the $ApoE^{-/-}$Angptl $4^{-/-}$ and $ApoE^{-/-}$ Angptl $4^{+/+}$ littermates used in this study. Mice had free access to regular chow and water during the study. All procedures were approved by the Animal Care and Use Committee of Kumamoto University (No. A19-039).

*Blood analysis.* Plasma total cholesterol, triglyceride (TG) and HDL cholesterol concentrations were measured using high-performance liquid chromatography (HPLC) by Skylight Biotech Inc. (Akita, Japan). Small dense LDL (sdLDL) was measured as particles under 25.5 nm of the LDL cholesterol fraction by HPLC.

*Intragastric lipid-loading test.* After an overnight fast, mice received 300 μl olive oil by oral gavage. Blood samples were drawn at baseline and various time points for up to 24 h after olive oil gavage. In the intragastric lipid-loading test to compare Angptl $4^{+/+}$ mice with Angptl $4^{-/-}$ mice, TG levels were enzymatically measured by Fuji Dry Chemistry (Fuji film, Tokyo, Japan). In the experiment to compare ApoE $^{-/-}$ Angptl $4^{+/+}$ mice with ApoE $^{-/-}$ Angptl 4 $^{-/-}$ mice, TG levels were enzymatically measured by Accutrend GCT (Roche Diagnostics K.K, Basel, Swiss). In the intragastric lipid-loading test with Triton WR 1339, 500 mg/kg body weight Triton WR 1339 as a 15% solution in 0.9% NaCl injection was administered intravenously, followed immediately by 300 μl olive oil by gavage. Serum lipoprotein clearance is completely inhibited under these circumstances [12]. Blood samples for TG levels were measured by HPLC at baseline and various time points up to 24 h after olive oil loading.

*Anti-atherogenic capabilities of Angptl 4 in vivo. ApoE $^{-/-}$ Angptl $4^{+/+}$ and ApoE $^{-/-}$ Angptl 4 $^{-/-}$ male mice were fed a normal rodent chow diet (Clea, Tokyo, Japan). Whole aorta were collected and stained with Sudan IV, and 6-μm-thick frozen cross sections of the aortic sinus were prepared and stained with oil red O according to the method described by SRL Inc. (Tokyo, Japan) [13]. The size of the atherosclerotic area was measured by BZ-II analysis software (Keyence, Osaka, Japan).*

*Experiments using cultured macrophages.* Peritoneal macrophages were collected and cultured from male mice as previously described [14].

*Lipoprotein preparation and modification of native LDL.* Human LDL ($d$ = 1.019–1.063 g/ml) and Oxidized-LDL was prepared as previously described [14]. Acetyl-LDL (acLDL) was prepared by chemical modification of LDL with acetic anhydride as previously described [15]. The peritoneal macrophages were incubated for 5 h at 37 °C with 50 μg/mL acLDL [13].

*Foam cell formation (CE-accumulation) assay.* The peritoneal macrophages were incubated with 50 μg/mL acLDL for 24 h in the presence of 0.1 mmol/L [$^3$H]oleate conjugated with BSA, and cellular lipids were extracted to determine the radioactivity of cholesteryl-[$^3$H]oleate as described previously [13].

*Real-time quantitative* PCR analysis. Real-Time Quantitative PCR was performed as described previously [14]. The following primer sequences were used in this study. Primer pairs for Angptl 4 and 36B4 were 5′-AGCTCAAGGCTCAAAACAGCA-3′, 5′-CTTTCCCCTCGAA GTCTTGTCT-3′; 5′-ATTGCCAGACGGAGCCG-3′, 5′-TCCTCATCTGAT TCCTCCGAC-3′, respectively.

*Statistical analyses.* Quantitative data are presented as means ± SEM of at least three independent experiments. Statistical analysis was based on Student's $t$-test for paired or unpaired data as appropriate. A $P$ value less than 0.05 was considered statistically significant.

## Results

### *Plasma cholesterol and lipoprotein profiles in Angptl $4^{-/-}$ mice and ApoE $^{-/-}$ Angptl 4 $^{-/-}$ mice*

We first analyzed plasma cholesterol and lipoprotein profiles in Angptl $4^{+/+}$ and Angptl $4^{-/-}$ mice. Fasting serum T-CHO, TG, very-low-dense lipoprotein (VLDL-C), low-dense lipoprotein (LDL-C) and high-dense lipoprotein (HDL-C) were significantly lower in Angptl 4 $^{-/-}$ mice compared with Angptl $4^{+/+}$ mice fed the normal diet (Fig. 1A).

The ApoE $^{-/-}$ Angptl 4 $^{-/-}$ mice, which we generated, had no abnormalities of general appearance and showed comparable, survival rate, food intake and body weight gain compared with $ApoE^{-/-}$ mice fed the normal diet (data not shown). In $ApoE^{-/-}$Angptl $4^{-/-}$ mice, fasting T-CHO and VLDL-C levels were not changed compared with $ApoE^{-/-}$Angptl $4^{+/+}$ mice. However, LDL-C, HDL-C and TG were significantly lower in ApoE $^{-/-}$ Angptl 4 $^{-/-}$ mice compared with $ApoE^{-/-}$Angptl $4^{+/+}$ mice fed the normal diet (Fig. 1B). Overall, the reduced LPL activity caused the elevated small dense LDL (sdLDL) [16]. On the other hand, some reports indicate that sdLDL is atherogenic and overexpression of LPL in transgenic rabbits led to significantly increased sdLDL particles [17]. Our data showed that sdLDL measured by HPLC was significantly lower in Angptl $4^{-/-}$ mice and $ApoE^{-/-}$Angptl $4^{-/-}$ mice compared with Angptl $4^{+/+}$ and $ApoE^{-/-}$Angptl $4^{+/+}$ mice, respectively (Fig. 1C). These results suggest that plasma cholesterol and lipoprotein profiles are improved not only in Angptl 4 $^{-/-}$ mice, but also in $ApoE^{-/-}$Angptl $4^{-/-}$ mice during normal diet feeding.

### *Postprandial TG response in Angptl $4^{-/-}$ and $ApoE^{-/-}$Angptl $4^{-/-}$ mice*

As postprandial hypertriglyceridemia is considered to be one of the risk factors of atherosclerosis, we measured the plasma TG concentration after oral administration of 300 μl olive oil to estimate the postprandial TG response. While Angptl $4^{+/+}$ mice showed a significant TG increase upon olive oil challenge, the serum TG response was almost blunted in Angptl $4^{-/-}$ mice (Fig. 2A). ApoE $^{-/-}$Angptl $4^{-/-}$ mice showed a similar phenotype on the olive oil challenge test (Fig. 2B). To further test the possible participation of LPL, mice received an intravenous injection of 500 mg/kg Triton WR 1339 to inhibit LPL activity (Fig. 2C). Angptl $4^{-/-}$ mice showed increased TG levels by inhibiting LPL using Triton WR 1339 after an oral administration of oil, but Angptl $4^{-/-}$ mice without Triton WR 1339 did not show increased serum TG concentrations. These results suggest that the accelerated LPL activity contributes to the suppressed TG in Angptl $4^{-/-}$ and $ApoE^{-/-}$Angptl $4^{-/-}$ mice.

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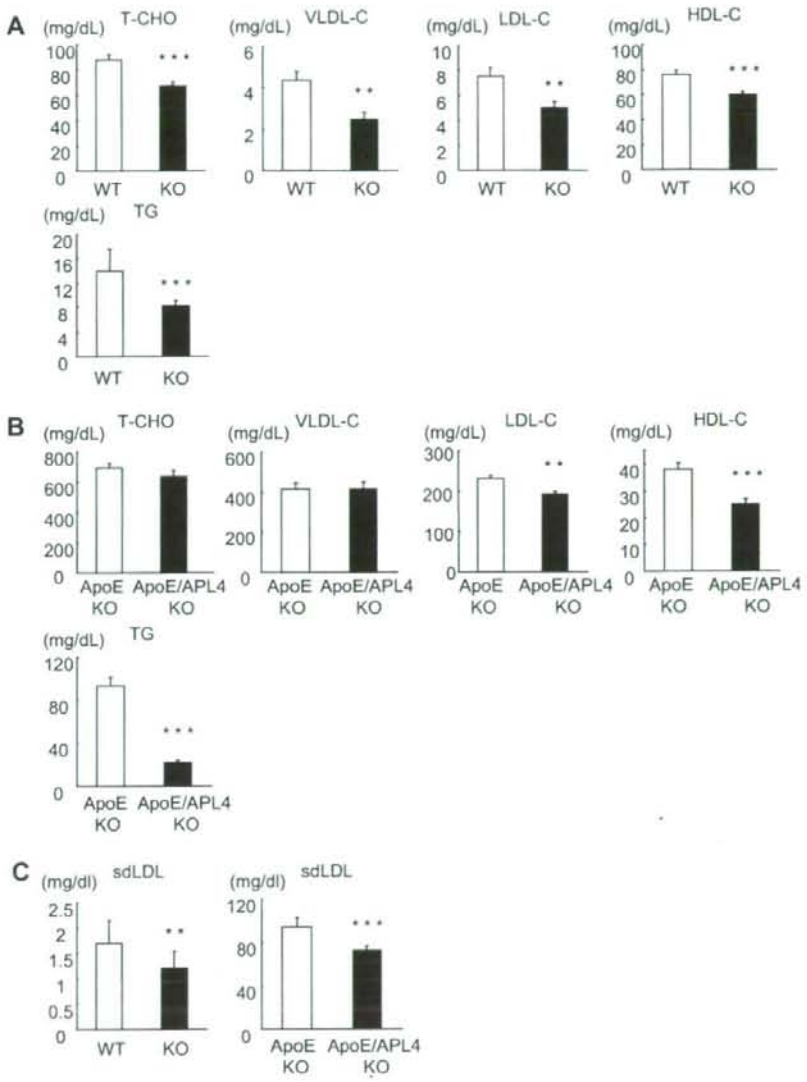


**Fig. 1.** Plasma Cholesterol and Lipoprotein Profiles in Angptl 4 $^{-/-}$ mice and ApoE $^{-/-}$ Angptl 4 $^{-/-}$ mice. HPLC analysis of plasma lipoproteins. Plasma samples were obtained after 16 h of fasting from male mice of each genotype fed a normal diet. (A,B) The T-CHO, VLDL-C, LDL-C, HDL-C and TG are shown for 12- to 16-week-old Angptl 4$^{+/+}$ mice (WT) ($n$ = 15), 12- to 16-week-old Angptl 4$^{-/-}$ mice (KO) ($n$ = 14), 12- to 13-week-old ApoE$^{-/-}$ Angptl 4$^{+/+}$ mice (ApoE KO) ($n$ = 8) and 12- to 13-week-old ApoE$^{-/-}$ Angptl 4$^{-/-}$ mice (ApoE/APL4 KO) ($n$ = 9). (C) sdLDL was measured in the fasting state by HPLC (WT group, $n$ = 5; KO group, $n$ = 5; ApoE KO group, $n$ = 8; ApoE/APL4 KO group, $n$ = 9). Data are presented as means ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ assessed by $t$-test.

*The absence of Angptl 4 restricts development of atherosclerosis on an ApoE $^{-/-}$ genetic background*

The development of atherosclerosis was examined in ApoE$^{-/-}$Angptl 4$^{+/+}$ and ApoE$^{-/-}$Angptl 4$^{-/-}$ male mice at 12 weeks of age (Fig. 3A and B). There was a significant 75 ± 12% reduction in atherosclerotic lesion size in ApoE$^{-/-}$Angptl 4$^{-/-}$ mice compared with ApoE $^{-/-}$ Angptl 4$^{+/+}$ mice. The atherosclerotic lesion visualized by Zudan IV staining was also reduced in ApoE $^{-/-}$ Angptl 4$^{-/-}$ mice at 16 weeks (Fig. 3C). Therefore, the absence of Angptl 4 could protect against atherosclerosis development in an ApoE $^{-/-}$ genetic background under normal diet feeding.

*The absence of Angptl 4 suppresses foam cell formation*

Foam cell formation is characteristic of atherosclerosis [18] and the absence of Angptl 4 protected against atherosclerosis development in male ApoE $^{-/-}$ mice. To further examine the impact of Angptl 4 knockout on atherosclerosis, we assessed foam cell formation from peritoneal macrophages isolated from mice as an

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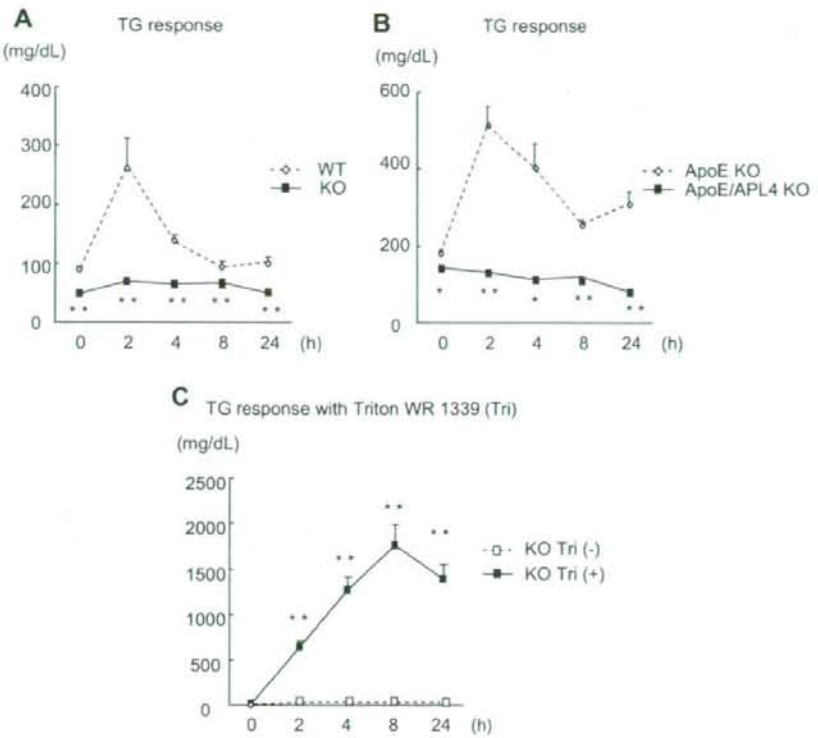


**Fig. 2.** TG response after olive oil loading in Angptl 4 $^{-/-}$ and ApoE $^{-/-}$ Angptl 4 $^{-/-}$ mice. (A,B) Serum TG levels were measured at baseline (0), 2, 4, 8 and 24 h after oral administration of 300 μl olive oil in 16-h-fasted Angptl 4$^{-/-}$ mice and Angptl 4$^{+/+}$ mice (WT group, $n$ = 9; KO group, $n$ = 12; ApoE KO group, $n$ = 5; ApoE/APL4 KO group, $n$ = 5). (C) KO (16-h-fasted, $n$ = 5) mice were treated with or without an intravenous injection of 500 mg/kg Triton WR 1339 (Tri) to inhibit LPL activity; serum TG was measured at baseline (0) and 2, 4, 8 and 24 h after 300 μl olive oil gavage. Data are presented as means ± SEM. $^{*}P < 0.01$ and $^{**}P < 0.001$ assessed by $t$-test.

*in vitro* model. Before the experiment, we confirmed the absence of Angptl 4 mRNA expression in peritoneal macrophages isolated from Angptl 4$^{-/-}$ mice by quantitative RT-PCR (data not shown). As shown in Fig. 4A, Angptl 4 deficiency reduced the accumulation of oil red O after incubation for 3 days with oxidized low-density lipoprotein (oxLDL) (Fig. 4A). Furthermore, to quantify cholesteryl ester (CE) formation, we cultured peritoneal macrophages with acetylated low-density lipoprotein (acLDL), measured the incorporation of [$^3$H]oleic acid to the CE pool, and assessed the accumulation of newly synthesized CE. In this experiment, the macrophages isolated from Angptl 4$^{-/-}$ mice showed a significant decrease in newly synthesized CE accumulation induced by acLDL compared with the macrophages isolated from Angptl 4$^{+/+}$ mice (Fig. 4B). These results suggest that Angptl 4 deficiency has a significant inhibitory effect on foam cell formation.

## Discussion

Recently, Angptl 4 has been shown to regulate lipid metabolism mainly by the inhibition of LPL activity [7–10]. However, it is still unknown whether Angptl 4 affects the development and/or progression of atherosclerosis *in vivo*.

In this study, we demonstrated for the first time that Angptl 4 deficiency protects against the development and progression of atherosclerosis in ApoE$^{-/-}$ mice. We characterized three underlying mechanisms of this phenomenon. First, serum lipid metabolism was improved in Angptl 4$^{-/-}$ mice and ApoE$^{-/-}$Angptl 4$^{-/-}$ mice. Second, postprandial hypertriglyceridemia was suppressed in Angptl 4$^{-/-}$ mice and ApoE$^{-/-}$Angptl 4$^{-/-}$ mice. Third, macrophages isolated from Angptl 4$^{-/-}$ mice showed reduced foam cell formation.

In this study, we used the Angptl 4 $^{-/-}$ mice previously reported by Bäckhed et al. [8]. To generate the mice, endogenous 5′-flanking region of Angptl 4 was replaced by a βgeopA/pGKneo cassette. Several researchers have reported the phenotypes of several lines of Angptl 4$^{-/-}$ mice in terms of lipid metabolism. Anja Koster et al. generated Angptl 4$^{-/-}$ mice using a targeting vector, in which a 2.05-kb region of the Angptl 4 gene including most of exon 1 containing the start codon and the signal peptide and exons 2 and 3 was deleted [10]. In the Angptl 4$^{-/-}$ male mice developed in that study, plasma TG levels were reduced by nearly 90% compared with their wild-type controls. Desai et al. also reported Angptl 4$^{-/-}$ mice generated from OmniBank ES cell clone OST352973, which contains a gene-trapping vector insertion in the second intron of Angptl 4 [9]. Their Angptl4$^{-/-}$ mice showed lower TG levels resulting from increased VLDL clearance and decreased VLDL production, and had modestly lower cholesterol levels. A similar phenotype is observed in mice in which Angptl 4 is inactivated using a monoclonal antibody against Angptl 4$^{-/-}$ [9].

Impaired lipid metabolism is well established as one of the major treatable risk factors for coronary heart disease. It is usually assessed in terms of the fasting levels of atherogenic LDL and anti-atherogenic HDL. In addition to the fasting dyslipidemia, "Postprandial hypertriglyceridemia" has been proposed as an alter-

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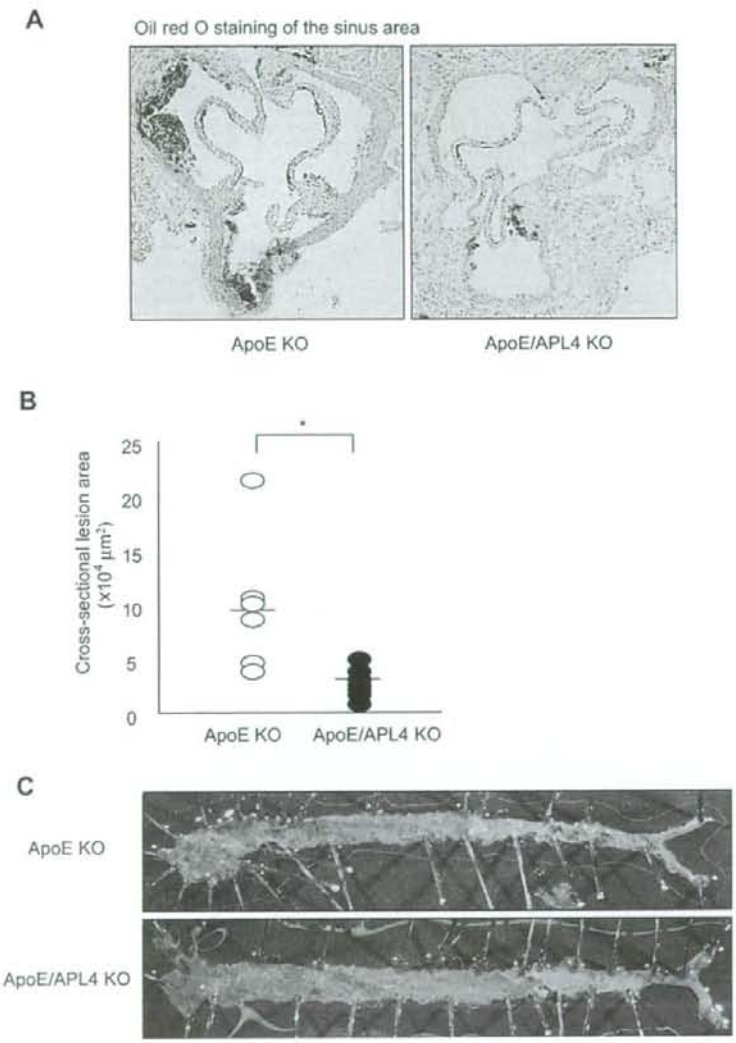


**Fig. 3.** Atherosclerotic analysis of ApoE $^{-/-}$ Angptl 4 $^{-/-}$ mice. (A,B) Computer-assisted morphometric measurement and representative sinus sections of oil red O staining of atherosclerotic lesion areas of 12-week-old male ApoE/APL4 KO ($n = 6$) and ApoE KO ($n = 7$) mice fed the normal diet. (C) Pinned-out aortas showing Sudan-IV-positive lesions of 16 weeks male ApoE KO and ApoE/APL4 KO mice fed the normal diet. Data are presented as means ± SEM. $^{*}P < 0.03$, assessed by *t*-test.

native or additive explanation for the etiology of cardiovascular diseases [2]. In our study, Angptl $4^{-/-}$ mice and ApoE$^{-/-}$Angptl $4^{-/-}$ mice had improved lipid profiles and decreased TG response to the olive oil challenge compared with the control groups. According to our present results, inactivation of Angptl 4 might ameliorate atherosclerosis by inhibiting the excessive postprandial hypertriglyceridemia. In addition, dyslipidemia in insulin resistant patients is characterized by high levels of plasma TG, low levels of HDL, the appearance of sdLDL and excessive postprandial hypertriglyceridemia [19]. Thus targeted inhibition of Angptl 4 may offer a new therapeutic strategy for the treatment of dyslipidemia in diabetes and metabolic syndrome. A previous report revealed that the lipid profile of Angptl $4^{-/-}$ mice was highly predictive for the target-based effects of the anti-Angptl 4 mAb [9]. Our present study further adds novel evidence that the inhibition of Angptl 4 protects against the development and progression of atherosclerosis *in vivo*.

The lipid-laden macrophage or foam cell is a characteristic of atherosclerosis [18]. Although we did not measure LPL activity in peritoneal macrophages, several reports have indicated that LPL is expressed in macrophages of atherosclerotic lesions, suggesting that LPL is proatherogenic [20]. However, in this study, we found that Angptl 4 plays a key role in foam cell formation, and its absence suppressed the ability of the macrophages to become foam cells *in vitro*.

Recent work suggests that Angptl 4 may play a similar role in humans, because individuals carrying the Angptl 4 E40K variant have significantly lower TG levels and higher HDL levels compared with wild-type E40E subjects [21]. On the other hand, Angptl $4^{-/-}$ mice showed lower TG levels but lower plasma HDL compared

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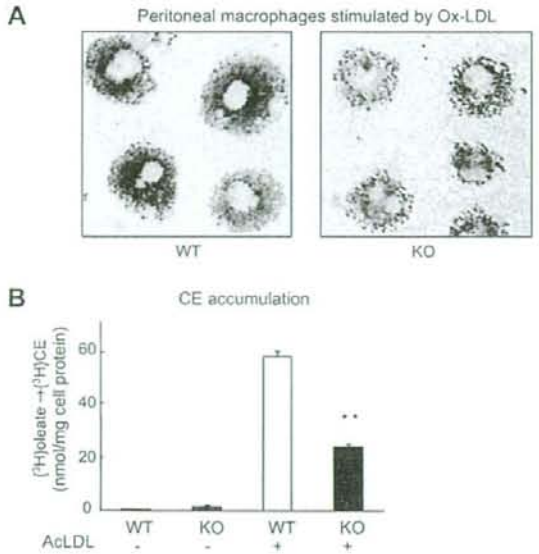


**Fig. 4.** Foam Cell Formation analysis of peritoneal macrophages isolated from Angptl $4^{-/-}$ mice. (A) Oil red O staining of peritoneal macrophages stimulated by oxidized LDL (oxLDL). Peritoneal macrophages isolated from mice were incubated with oxLDL. (B) Peritoneal macrophages were incubated with acetyl-LDL (acLDL) and [$^3$H] CE was measured (WT group, $n = 10$; KO group, $n = 10$). Data are presented as means ± SEM. $^{*}P < 0.05$ and $^{**}P < 0.001$ assessed by *t*-test.

with control mice, the mechanism of which is not clear. A recent report suggested that Angptl 3 acts as an inhibitor of endothelial lipase, which may be involved in the regulation of plasma HDL [22]. Angptl 4 may also act as an inhibitor of endothelial lipase.

In summary, we have shown that serum LDL, HDL and TG levels are decreased in Angptl $4^{-/-}$ mice and ApoE$^{-/-}$Angptl $4^{-/-}$ mice. In addition, postprandial hypertriglyceridemia was improved in Angptl $4^{-/-}$ mice and ApoE$^{-/-}$Angptl $4^{-/-}$ mice. Angptl 4 deficiency protected against atherosclerosis in ApoE$^{-/-}$ mice. Macrophages isolated from Angptl $4^{-/-}$ mice showed decreased foam cell formation. Therefore, the targeted silencing of Angptl 4 offers a potential therapeutic strategy for the treatment of dyslipidemia and atherosclerosis.

## Acknowledgments

We appreciate the helpful advice and assistance of Mr. Kenshi Ichinose in the Department of Metabolic Medicine of Medical and Pharmaceutical Sciences, Kumamoto University. RT-PCR primers for Angptl 4 were provided by Kunio Yasunaga (Department of Cardiology, Astellas Pharmaceutical Co. Ltd., Tsukuba, Japan). This work was supported by a grants-in-aid for Scientific Research from the Japan Society for the Promotion of Science, Japan (Nos. 16046219 and 20390259 to Eiichi Araki), a grant from the Ministry of Education, Science, Sports and Culture of Japan (No. 19591058 to Tatsuya Kondo) and a grant from Suzuken Memorial Foundation (to Tatsuya Kondo).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 283, NO. 15, pp. 9852–9862, April 11, 2008


# Oxidized Low Density Lipoprotein Activates Peroxisome Proliferator-activated Receptor-α (PPARα) and PPARγ through MAPK-dependent COX-2 Expression in Macrophages*

Received for publication, April 20, 2007, and in revised form, December 26, 2007 Published, JBC Papers in Press, January 21, 2008, DOI 10.1074/jbc.M703318200

**Kayo Taketa‡, Takeshi Matsumura‡1, Miyuki Yano‡, Norio Ishii‡, Takafumi Senokuchi‡, Hiroyuki Motoshima‡, Yusuke Murata‡, Shokei Kim-Mitsuyama§, Teruo Kawada¶, Hiroyuki Itabe‖, Motohiro Takeya**, Takeshi Nishikawa‡, Kaku Tsuruzoe‡, and Eiichi Araki‡**

*From the ‡Department of Metabolic Medicine, §Department of Pharmacology and Molecular Therapeutics, and **Department of Cell Pathology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 861-8556, the ¶Laboratory of Nutrition Chemistry, Division of Food Science and Biotechnology, Graduate School of Agriculture, Kyoto University, Gokasyou, Uji, Kyoto 611-0011, and the ‖Department of Biological Chemistry, School of Pharmaceutical Sciences, Showa University, 1-5-8 Hatanodai, Shinagawa-ku, Tokyo 142-8555, Japan*

**It has been reported that oxidized low density lipoprotein (Ox-LDL) can activate both peroxisome proliferator-activated receptor-α (PPARα) and PPARγ. However, the detailed mechanisms of Ox-LDL-induced PPARα and PPARγ activation are not fully understood. In the present study, we investigated the effect of Ox-LDL on PPARα and PPARγ activation in macrophages. Ox-LDL, but not LDL, induced PPARα and PPARγ activation in a dose-dependent manner. Ox-LDL transiently induced cyclooxygenase-2 (COX-2) mRNA and protein expression, and COX-2 specific inhibition by NS-398 or meloxicam or small interference RNA of COX-2 suppressed Ox-LDL-induced PPARα and PPARγ activation. Ox-LDL induced phosphorylation of ERK1/2 and p38 MAPK, and ERK1/2 specific inhibition abrogated Ox-LDL-induced COX-2 expression and PPARα and PPARγ activation, whereas p38 MAPK-specific inhibition had no effect. Ox-LDL decreased the amounts of intracellular long chain fatty acids, such as arachidonic, linoleic, oleic, and docosahexaenoic acids. On the other hand, Ox-LDL increased intracellular 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) level through ERK1/2-dependent overexpression of COX-2. Moreover, 15d-$PGJ_2$ induced both PPARα and PPARγ activation. Furthermore, COX-2 and 15d-$PGJ_2$ expression and PPAR activity were increased in atherosclerotic lesions of apoE-deficient mice. Finally, we investigated the involvement of PPARα and PPARγ on Ox-LDL-induced mRNA expression of ATP-binding cassette transporter A1 and monocyte chemoattractant protein-1. Interestingly, specific inhibition of PPARα and PPARγ suppressed Ox-LDL-induced ATP-binding cassette transporter A1 mRNA expression and enhanced Ox-LDL-induced monocyte chemoattractant protein-1 mRNA expression. In conclusion, Ox-LDL-induced increase in 15d-$PGJ_2$ level through ERK1/2-dependent COX-2 expression is one of the mechanisms of PPARα and PPARγ activation in macrophages. These effects of Ox-LDL may control excess atherosclerotic progression.**

Accumulation of modified low density lipoprotein (LDL),[2] such as oxidized LDL (Ox-LDL), and recruitment of monocytes in the arterial subendothelial spaces are early events in atherogenesis (1). Macrophages, which are derived from monocytes in these areas, take up Ox-LDL through the scavenger receptor pathways and become foam cells (2). Foam cells are well known to play an important role in the development and progression of atherosclerosis, through the production of various bioactive molecules such as growth factors and cytokines (1).

Peroxisome proliferator-activated receptors (PPARs) are transcription factors belonging to the nuclear receptor supergene family (3–5). Three distinct PPARs, termed α, δ, and γ, have been identified. PPARs are characterized by distinct tissue distribution patterns and metabolic functions. PPARα is highly expressed in tissues that demonstrate high catabolic rates for fatty acids, such as liver, heart, kidney, and muscle, whereas PPARγ is highly expressed in adipose tissue where it plays a major regulatory role in adipocyte differentiation, and the expression of genes involved in lipid metabolism (4–7). PPARδ shows a widespread tissue distribution, but its physiological role remains to be fully elucidated (8). Recently, agonists of PPARα and PPARγ have been reported to improve atherosclerosis in LDL receptor-deficient mice (9, 10), suggesting that activation of PPARα and/or PPARγ suppresses the development and progression of atherosclerosis.

Recently, we have reported that 3-hydroxyl-3-methylglutaryl-CoA reductase inhibitors (statins) induced activation of PPARα and PPARγ in macrophages (11). Moreover, we revealed that statin-induced activation of PPARα and PPARγ were mediated by cyclooxygenase-2 (COX-2)-dependent

* This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Japan (Grant 20398192 to T. M.), by a grant from the Takeda Science Foundation (to T. M.), and by a grant from the Japan Diabetes Foundation (to T. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] To whom correspondence should be addressed. Tel.: 81-96-373-5169; Fax: 81-96-366-8397; E-mail: takeshim@gpo.kumamoto-u.ac.jp.

[2] The abbreviations used are: LDL, low density lipoprotein; ABCA1, ATP-binding cassette transporter A1; COX-2, cyclooxygenase-2; ERK1/2, extracellular signal-regulated kinase 1/2; MAPK, mitogen-activated protein kinase; MCP-1, monocyte chemoattractant protein-1; 15d-$PGJ_2$, 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$; PPAR, peroxisome proliferator-activated receptor; 9-HODE, 9-hydroxyoctadecadienoic acid; 13-HODE, 13-hydroxyoctadecadienoic acid; ERK, extracellular signal-regulated kinase; LPS, lipopolysaccharide; WT, wild type; EIA, enzyme immunoassay; TG, triglyceride; m-Ox-LDL, mildly oxidized LDL; c-LDL, control LDL; Mm-Ox-LDL, macrophage-mediated Ox-LDL; C/EBP, CAAT/enhancer-binding protein; TBARS, thiobarbituric acid-reactive substances; CMV, cytomegalovirus; siRNA, small interference RNA; RT, reverse transcription.

increase in intracellular 15-deoxy-$\Delta^{12,14}$-prostaglandin $J_2$ (15d-$PGJ_2$) production (11). Therefore, several pathways that lead to the overexpression of COX-2 may also induce the activation of PPARα and PPARγ.

Ox-LDL has been reported to activate PPARα in endothelial cells (12, 13) and PPARγ in CV-1 monkey kidney fibroblasts (14), and this is possibly caused by the action of oxidized metabolites of linoleic acid, including 9-hydroxyoctadecadienoic acid (9-HODE) and 13-hydroxyoctadecadienoic acid (13-HODE), and oxidized phospholipids, which are included in Ox-LDL (12, 14). However, the detailed mechanism involved in Ox-LDL-induced activation of PPARs and the role of PPARs in Ox-LDL-mediated acceleration of atherogenesis are not fully understood.

In the present study, we investigated a novel mechanism of Ox-LDL-induced activation of PPARs in macrophages. We demonstrated that Ox-LDL activated both PPARα and PPARγ through extracellular signal-regulated kinase 1/2 (ERK1/2)-dependent COX-2 expression in macrophages. In addition, Ox-LDL-induced activation of PPARs mediated the induction of ATP-binding cassette transporter A1 (ABCA1) mRNA expression, as well as the suppression of monocyte chemoattractant protein-1 (MCP-1) mRNA expression.

## EXPERIMENTAL PROCEDURES

*Materials*—PD98059, SB203580, meloxicam, NS-398, T0070907, and 15d-$PGJ_2$ were purchased from Calbiochem. Lipopolysaccharide (LPS) (*Escherichia coli* O111:B4) and GW6471 were purchased from Sigma. Rabbit polyclonal anti-ERK1/2, anti-p38 mitogen-activated protein kinase (MAPK), anti-PPARα and anti-PPARγ antibodies, and goat polyclonal anti-β-actin, anti-integrin αM, and anti-apolipoprotein-B (apoB) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit polyclonal anti-phospho ERK1/2 and anti-phospho p38 MAPK antibodies were purchased from Cell Signaling Technology (Beverly, MA). A rabbit polyclonal anti-murine COX-2 antiserum was purchased from Cayman Chemical (Ann Arbor, MI). All other chemicals were of the best grade available from commercial sources.

*Animals*—C57BL/6 mice and apolipoprotein E-deficient ($apoE^{-/-}$) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). These mice were maintained on the C57BL/6 background strain, and C57BL/6 mice were used as wild-type (WT) mice. Mice were given access to food and water *ad libitum* in the Animal Resource Facility at Kumamoto University under specific pathogen-free conditions. All animal procedures were approved by the Animal Research Committee at Kumamoto University, and all procedures conformed to the Guide for the Care and Use of Laboratory Animals issued by the Institute of Laboratory Animal Resources. The diet was a normal rodent chow diet for mouse (CLEA, Tokyo, Japan). 40 male mice of 24 weeks of age (20 as C57BL/6 and 20 as $apoE^{-/-}$) were sacrificed, and atherosclerotic lesions of aortic sinus were used for Western blot assay, enzyme immunoassay (EIA) for 15d-$PGJ_2$, assay of transcription activity of PPARα and PPARγ, and immunohistochemistry as described below. Plasma total cholesterol, triglyceride (TG), and HDL cholesterol concentrations were performed commercially by Skylight Biotech Inc. (Akita, Japan).

*Immunohistochemistry*—Atherosclerotic lesion at aortic sinus was obtained from 24-week-old $apoE^{-/-}$ mice. 6-μm-thick frozen sections of aortic sinus obtained from $apoE^{-/-}$ and WT mice were stained with oil red O as described (15). For immunohistochemistry, 3-μm-thick serial paraffin sections were used. First antibodies used were biotinylated FOH1a/DLH3 (16) for Ox-LDL and goat polyclonal anti-integrin αM (Santa Cruz Biotechnology) for CD11b. As the second step reaction, peroxidase-conjugated streptavidin (Nichirei, Tokyo, Japan) for Ox-LDL and Hitofine Simple Stain Mouse MAX-PO (Goat, Nichirei) for CD11b were used. After visualization of peroxidase activity using 3,3-diaminobenzidine as a substrate, the sections were stained with hematoxylin for nuclear staining. As negative controls, the same procedures were performed, but the primary antibodies were omitted.

*Cell Cultures*—Peritoneal macrophages were collected from anesthetized male C3H/He mice (25–30 g body weight) by peritoneal lavage with 8 ml of phosphate-buffered saline, centrifuged at 200 × *g* for 5 min, resuspended in medium A (RPMI 1640 medium (Nissui Seiyaku, Tokyo, Japan) supplemented with 10% fetal calf serum (Invitrogen), 0.1 mg/ml streptomycin, and 100 units/ml penicillin), and incubated in appropriate tissue culture plates for 90 min (17). More than 98% of the adherent cells were considered to be macrophages, based on four criteria, as described previously (18, 19). RAW264.7 cells were cultured in medium A in appropriate tissue culture plates as previously described (20) and used from passage numbers four to eight.

THP-1 cells, a human monocytic cell line, were cultured in medium A in 35-mm dishes and treated with 200 nM phorbol myristate acetate to induce their differentiation into macrophages. After 8 h of incubation, the cells were washed twice with phosphate-buffered saline and incubated in medium A until the initiation of experiments (11).

*Lipoprotein Preparation and Modification of Native LDL*—Human LDL ($d$ = 1.019–1.063 g/ml) was isolated by ultracentrifugation from the plasma of consenting normolipidemic subjects, obtained after overnight fasting (21). LDL was dialyzed against 0.15 M NaCl and 1 mM EDTA, pH 7.4. Ox-LDL or mildly oxidized LDL (m-Ox-LDL) was prepared by incubation of LDL with 5 μM $CuSO_4$ for 20 or for 5 h, respectively, at 37 °C, followed by the addition of 1 mM EDTA, and cooling (22). The concentration of proteins was determined by BCA protein assay reagent (Pierce). The endotoxin level of Ox-LDL was <1 pg/μg of protein measured by Toxicolor system (Seikagaku, Japan). Macrophage-mediated oxidation of LDL was performed by using Lindstedt's methods (23). Briefly, 100 μM LDL and 100 nM $CuSO_4$ were incubated at 37 °C with medium A in the absence (c-LDL)) or presence (Mm-Ox-LDL) of mouse peritoneal macrophages ($1 \times 10^6$). After 20-h incubation, the supernatant was removed, and the extent of lipid peroxidation of LDL in the supernatant was determined by measuring the amount of thiobarbituric acid-reactive substances (TBARSs) as described below.

*Analysis of Oxidation, Electrophoretic Mobility, and Degradation of apoB in LDL Modified with $CuSO_4$*—Following oxidative modification of LDL with $CuSO_4$ or macrophages, lipid peroxidation was assessed by the following procedures. The