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Progression of Atherosclerosis and Femoral Arterial Blood Pressure in Heritable Hypertriglyceridemic Rabbits

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ABSTRACT

[Objective] It has been reported that hypercholesterolemia plays a role in progression of atherosclerosis. Watanabe heritable hyperlipidemic rabbits have been used widely as a model of familial hypercholesterolemia with atherosclerosis, while the emerging data raised a possibility of an important role of hypertriglyceridemia in the pathogenesis of atherosclerosis. We have recently segregated a new line with severely high (TGH) and moderately high (TGL) levels of plasma triglyceride. The hemodynamic parameters of TGH and TGL are not defined. The aim of present study was to examine the progression of atherosclerosis and hemodynamic parameters of TGH.

[Methods] Japanese White rabbits (JW) and TGH were anesthetized with ketamine and xylazine. BP was measured by a catheter implanted in the femoral artery. Histological examination was carried out with Elastica-Masson trichrome staining to detect atherosclerotic lesions.

[Results] JW had no atherosclerotic lesions. In TGH, severe atherosclerotic lesions were observed in the aortic arch. The basal femoral arterial pressure was not significantly different between JW and TGH. However, the basal pulse pressure in TGH was significantly greater than that of JW. Intravenous injection of N^G-nitro-L-arginine methyl ester (L-NAME) increased the blood pressure of TGH as well as JW. There was no difference in the response to L-NAME. The greater pulse pressure in TGH may be due to the increased vascular stiffness with atherosclerosis.

Key words : hyperlipidemia, triglyceride, atherosclerosis , blood pressure, NO

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実験技術

食後高トリグリセリド血症家兎(PHT)の開発経緯と生活習慣病モデルとしての有用性

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要約：血管病対策は21世紀の医療の重要な課題であり，血管病の原因は動脈硬化性変化である．動脈硬化の危険因子としては高コレステロール血症のほうが臨床的に重大である．しかし，虚血性心疾患を発症した全ての患者に高コレステロール血症を認めるわけではなく，むしろ他の危険因子（高トリグリセリド（TG）血症，耐糖能異常，肥満，高血圧等）を同一人が複数併せ持つ方が，虚血性心疾患を発症する頻度が高いことが報告されており，特に高トリグリセリド血症と虚血性心疾患との関係が注目されている．さらに，動脈硬化症を主要所見とする臨床例の中に，食後高脂血症を示す症例が知られており，血管病進展の重篤な危険因子と考えられている．著者らは，動脈硬化に対する血中のコレステロール高値の要因を排除するために，通常（絶食状態）は血中のコレステロール（CHO）とトリグリセリド（TG）が低値であり，食後にTGのみが異常高値を示す家兎（PHT: Postprandial Hyper Triglyceridemia，食後高トリグリセリド血症家兎）の分離に成功した．PHTはCHO代謝異常に依存しない新しい脂質代謝異常のモデル動物である．PHTの食後高トリグリセリド血症は，絶食時に100 mg/dl以下を示し，食後12～24時間で1,000 mg/dl以上のTG値を示す．制限食給餌（120 g/day）下でも食後TG値は1,000 mg/dlを超える．PHTの食後高トリグリセリド血症の発症時期は6ヵ月齢前後から顕著となる．また，PHTの雄性でより食後高トリグリセリド血症が顕著である．PHTの食後の血漿をリポタンパク分析した結果，VLDLの増加が顕著であった．PHTに糖負荷試験を行ったところ，耐糖能異常並びにインスリン抵抗性を認めた．PHTは野生型家兎（日本白色家兎）に比較して体型がやや小型だが，腹腔内脂肪の沈着が顕著である．

1. はじめに

生活習慣病の死因は癌（悪性新生物），心臓病（虚血性心疾患），脳血管障害であり，心臓病と脳血管障害を合わせると癌死を越える．したがって，血管病対策は21世紀の医療の重要な課題である．

血管病の原因は動脈硬化性変化である．地域住民を対象とした疫学調査によって動脈硬化症の発症と重症度を規定する危険因子が明らかとなり，その代表的なものは高脂血症，高血圧，糖尿病，喫煙，性（男性，閉経後の女性）である．高脂血症には高コレステロール血症と高トリグリセリド（中性脂肪）血症，それらの合併したものとがある．動脈硬化の危険因子としては高コレステロール血症のほうが臨床的に重大である．

しかしながら，欧米や日本の先進諸国では食事内容として年々高栄養価のものが摂取される傾向にあり，高トリグリセリド血症が問題視されるようになってきている．米国のFramingham研究によると，虚血性心疾患患者の35%は血清コレステロール（CHO）が200 mg/dl以下であったと報告している(1)．また，泰江らは心筋梗塞患者1,032例を対象として行ったJapanese Antiplatelets Myocardial Infarction Study（JAMIS）の結果において，220 mg/dl以上の高コレステロール血症を有する患者の割合はたった27.1%であったと報告している(2)．このように虚血性心疾患を発症した全ての患者に高コレステロール血症を認めるわけではなく，むしろ他の危険因子（高トリグリセリド（TG）血症，耐糖能異常，肥満，高血圧等）

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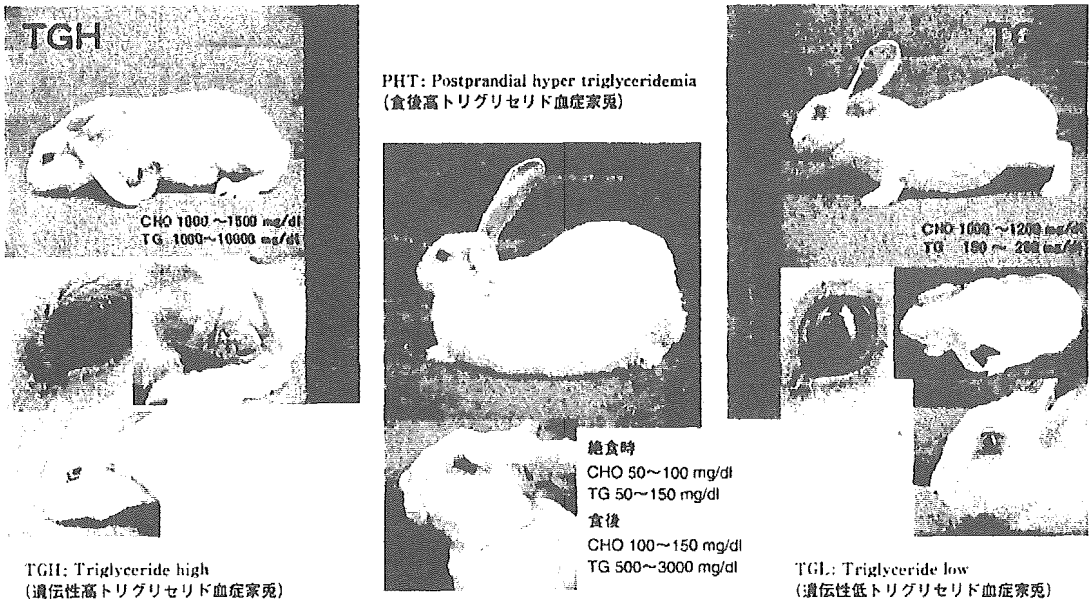

図1 山形大学で開発および維持されているウサギの系統

を同一人が複数併せ持つ方が，虚血性心疾患を発症する頻度が高いことが示され，特に高トリグリセリド血症と虚血性心疾患との関係が注目されている．さらに，動脈硬化症を主要所見とする臨床例の中に，食後高脂血症を示す症例が知られており，血管病進展の重篤な危険因子と考えられている．

しかし臨床例において，遺伝性の有無，脂質代謝の特徴は系統的に解析されていない．なぜ，高トリグリセリド血症が発生するのか，医学的な解明は進んでいない．その理由は，食事と高トリグリセリド血症の関係が未解決な事による．そのために，食後に高トリグリセリド血症を示す動物モデルの開発は，この問題の解明に必須と考えられる．

2. 遺伝性高トリグリセリド血症家兎(TGH)と遺伝性低トリグリセリド血症家兎(TGL)の系統確立

著者らはWatanabe heritable hyperlipidemic rabbit (WHHL) の分与を受け(3-5)，日本白色家兎 (JW) と戻し交配を行った後，ホモ接合体の確立を試みた．血清コレステロールと血清トリグリセリドを酵素法で測定したところ，WHHLのトリグリセリド値（中性脂肪）は200～900 mg/dlと幅広い分布を示す事が分かった．一方，野生型である日本白色家兎では血清トリグリセリド値は多くの場合100 mg/dl以下を示す．そこで，血清トリグリセリド値が高い個体の掛け合わせを行った結果，世代を追う毎に血清トリグリセリド値が500 mg/dlを超すWHHLの発現が増加した．即ち，遺伝性高トリグリセリド血症を示す家兎の発現頻度が上昇することが観察された．1995年の第4世代では90%，第5世代以降では100%の進達率で発現したことから，遺伝性高トリグリセリド血症家兎 (TGH) を系統として確立できた．同様に血清トリグリセリド値が低い個体の掛け合わせを行い，遺伝性低トリグリセリド血症家兎 (TGL) を系統として確立した．著者らはこれらの家兎を遺伝性高トリグリセリド血症家兎 (TGH，CHO＝1000～1500 mg/dl，TG＝1000～10000 mg/dl)，遺伝性低トリグリセリド血症家兎 (TGL, CHO＝1000 mg/dl, TG＝150～200 mg/gdl) と命名した (図1)．

WHHLの高コレステロール血症はLDL (low density lipoprotein：低比重リポタンパク) 受容体の異常であることが明らかにされているが，遺伝性高トリグリセリド血症家兎 (TGH) および遺伝性低トリグリセリド血症家兎 (TGL) はWHHL家兎亜系より選抜した家兎であり，WHHLの特徴であるコレステロール高値に加えトリグリセリドの異常高値および低値を特徴とする．

3. 食後高トリグリセリド血症家兎(PHT)の発見

著者らは，TGHの遺伝様式を検索する目的で，TGHと日本白色家兎 (JW) の交配により雑種第1世代を作出した．さらに雑種第1世代 (MHF1) の中での交

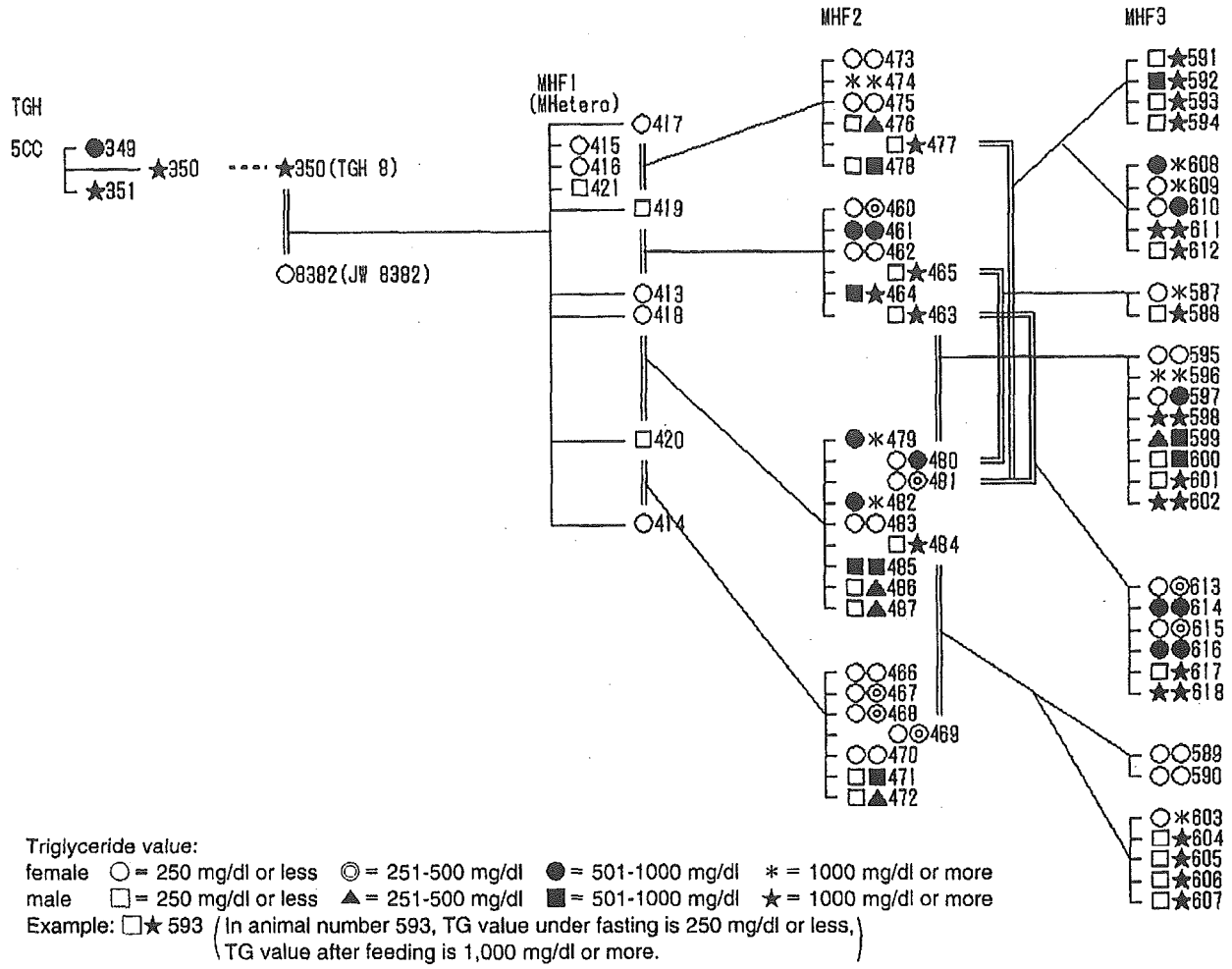

図2　PHTウサギの家系図

配〈兄妹交配／ヘテロ接合体どうしの交配なので，メンデルの法則から1/4はホモ接合体，1/4は野生型，2/4はヘテロ接合体が産まれる〉により第二世代（MHF2）を作出し，遺伝形質の解析を行ったところ，ホモ接合体以外の個体に食後高トリグリセリド血症家兎を見出した．すなわち，食餌摂取と血清脂質レベルの関係を検討したところ，空腹時には脂質レベルは正常であり，食後12時間以降に血清トリグリセリド値が1000 mg/dl以上になる特異な家兎の存在に気づいた．遺伝形質を調べたところ，食後高トリグリセリド血症は常染色体優性遺伝の形質を示唆することが分かった．図2に遺伝性食後高トリグリセリド血症家兎を作出した際の家系図を示した．WHHLと日本白色家兎との戻し交配により，第一世代〈MHF1〉および第二世代（MHF2）を作出した．MHF2においてはTGH 7匹，正常の脂質レベルを示す個体26匹を得た．その26匹の中で，食後に高トリグリセリド血症を示す7匹（雄4匹，雌3匹）を種動物として掛け合わせを行った結果，第三世代〈MHF3〉を，32匹（雄20匹，雌12匹）得た．その中で，食餌に関係なく血清高トリグリセリド血症を示すTGHの性質を有する個体は9匹，空腹時の脂質レベルは正常で食後に高トリグリセリド血症を示す個体は23匹であった．従って，TGHは約25%を占め，常染色体性劣性であることが確認された．食後に高トリグリセリド血症を示す個体は23匹だったことから，本形質はTGHと別の遺伝子支配による可能性が高く示唆された．そこで，本形質を示す家兎をモデル動物系として確立し，食後高トリグリセリド血症家兎（PHT: Postprandial Hyper Triglyceridemia）と命名し（図1），日本および米国の特許を取得した(6,7)．食後にのみ高トリグリセリド血症を示すという遺伝形質を有するモデル動物はこれまでに例がない．PHTはコレステロール代謝異常に依存しない新しいヒト虚血性心疾患のモデル動物とし

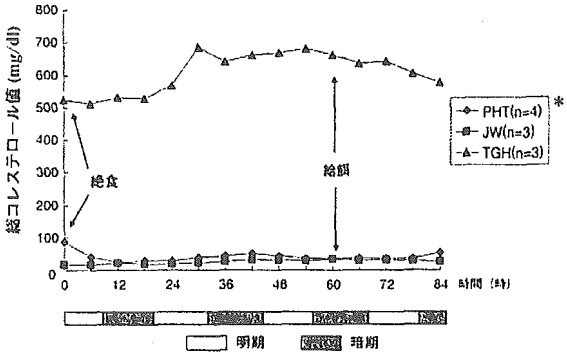

図3　食餌によるコレステロールの変動
＊PHT：食後高トリグリセリド血症家兎，JW：日本白色家兎，TGH：遺伝性高トリグリセリド血症家兎

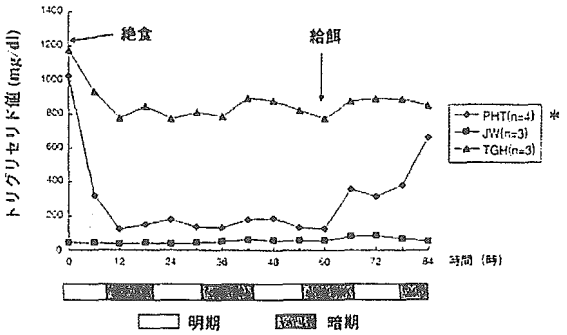

図4　食餌によるトリグリセリドの変動
＊PHT：食後高トリグリセリド血症家兎，JW：日本白色家兎，TGH：遺伝性高トリグリセリド血症家兎

て有用と考えられる．また，ヒトのマルチプルリスクファクター症候群や食後高脂血症の診断や解析に重要と考えられる．以下，PHTの特徴について述べる．

4. PHTにおける食後高トリグリセリド血症の特徴

PHTは，絶食時には10 mg dl以上200 mg dl以下の血清トリグリセリド値を示し，かつ食後12時間以後48時間以内に500 mg dl以上3000 mg dl以下の血清トリグリセリド値を示す事を特徴とする．

著者らが規定する高トリグリセリド血症とは500 mg dl以上のトリグリセリド値を示すことを意味し，1000～2000 mg dlをPHTの育種選抜目標としている．同様に食後とは食事開始後12～24時間後のことである．

各種血液脂質成分値を比較検討するためには，家兎の飼育条件，繁殖条件を一定にする必要がある．山形大学において作出維持されている家兎は温度22±2℃，湿度40-60%に管理された飼育室の中で，固型飼料（Labo R Grower，日本農産工業，東京）120 gを毎日一定量給餌されている．水は自由摂取である．飼育室の明暗は午前6時から午後6時までを照明時間としている．家兎の繁殖は自家で行い，娩出された家兎は生後30日で離乳された後に個別飼育された．生後1カ月齢から2カ月齢までの幼若家兎には80 gの固型飼料を毎日定量摂取させ，2ヵ月齢以後は120 gの制限給餌としている．

血液脂質の測定は，家兎の耳動脈から1 mlを採血し，1分間3000回転で15分間（4℃）の冷却遠心を行って血清または血漿を分離後，Vision Analyzer（Dinabot社，日本）を用いてコレステロールとトリグリセリドの測定を行った．著者らはPHTのスクリーニングとして，制限食を給餌した家兎に24～48時間の絶食を施した後に飽食給餌し，給餌開始24時間後の血漿トリグリセリド値を測定して評価している．

5. 野生型家兎（日本白色家兎，JW: Japanese White）との比較

PHTは，空腹時には血清トリグリセリド値は正常であるが，食後に高トリグリセリド血症を示すという特徴を有することから，ヒトの食後高脂血症のモデル動物として有用である．日本白色家兎（JW）では，食後のトリグリセリド増加は認められない．PHT，JWおよびTGHの絶食と食後の影響について血清コレステロールと血清トリグリセリドを指標に比較検討した．コレステロールは3系統とも特に変化を認めなかった（図3）．しかし，トリグリセリドは，JWとTGHでは絶食や食餌の影響を受けなかったのに対し，PHTでは，絶食前に約1000 mg/dlあったトリグリセリド値が，絶食開始後急激に低下し，12時間で約100 mg/dlを示した．絶食期間中は100～200 mg/dlで推移したが，その後食餌を与えるとトリグリセリド値は急激に上昇し，食後24時間で600 mg/dlを示した（図4）．このように，PHTは絶食と食餌に対してトリグリセリドの鋭敏な反応を示す(8)．

6. PHTの平時におけるトリグリセリド値

平時（制限食条件下で絶食等の前処置をしていない）におけるPHTの血清トリグリセリド値の経時変化を詳細に測定した（図5）．通常の給餌時間である昼の12時を食前（0時）とし，以後3時間おきに翌日の昼の12時まで24時間（0，3，6，9，12，15，18，21，24時）の血漿脂質成分値を測定した．すると食後6時間を過ぎる頃から血清トリグリセリド値は急速に上昇し，

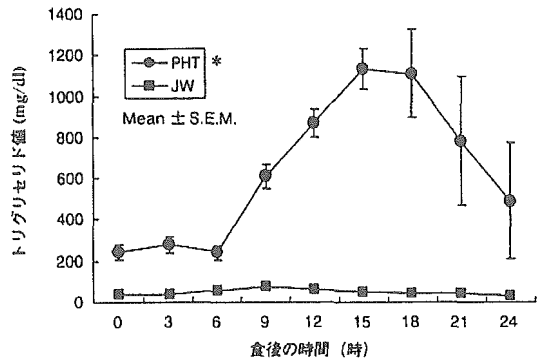

図5　PHTウサギの平時トリグリセリド値
＊PHT：食後高トリグリセリド血症家兎，JW：日本白色家兎

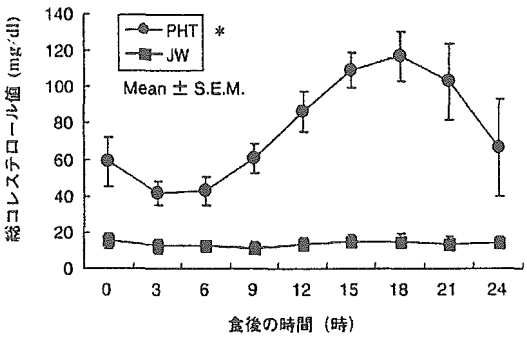

図6　PHTウサギの平時コレステロール値
＊PHT：食後高トリグリセリド血症家兎，JW：日本白色家兎

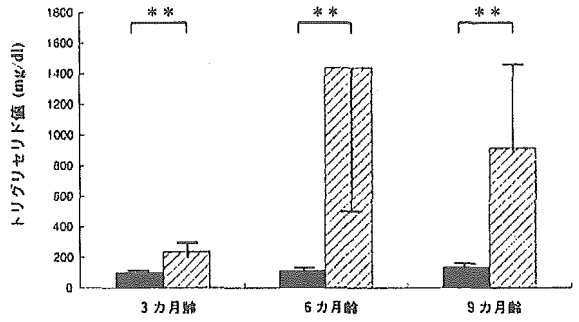

図7　PHTウサギの食後TG値における月齢差
■絶食，▨食後，平均値±標準偏差，n＝23（雄15，雌8），**P <0.01

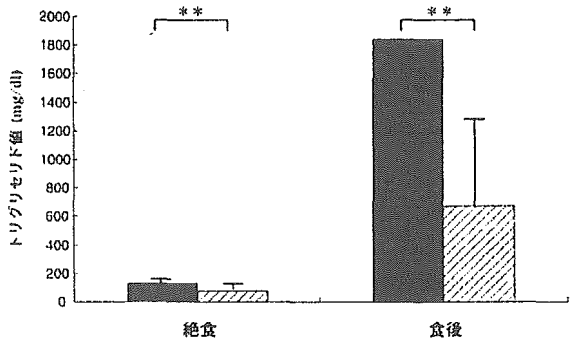

図8　PHTウサギの食後TG値における性差
■雄，▨雌，平均値±標準偏差，n＝23(雄15，雌8)，**P <0.01

食後18時間で血清トリグリセリド値は1000 mg dl以上という最高値に達した．野生型であるJWは，食事開始後6～9時間後にわずかなトリグリセリドの上昇(グラフからは判別つかないが)を認めるが，PHTのようなトリグリセリド値の極端な上昇は認められなかった．一方，PHTの平時における血清コレステロール値は，トリグリセリド値と同様の変化パターンを示したが，JWでは認められない（図6）．

7. PHTの食後高トリグリセリド血症の発症時期と性差

PHTの同一個体を用いて3，6，9カ月齢の食餌と血清脂質との関係を調べた．食餌の摂取量と食後のトリグリセリドレベルの間には有意の相関は認められなかった．従って，食後高トリグリセリド血症は食物の摂取と関連するが，食餌の摂取量は血液中の濃度を規定する因子ではないことになる．絶食時と食後の血清総コレステロール値の比較において，3カ月齢では有意の差を認めないが，6カ月齢では平均44 mg dlから118 mg dlへ，9カ月齢では41 mg dlから80 mg dlへと食餌により有意に増加した．血清トリグリセリド値を絶食時と食後で比較したところ，3カ月齢で103 mg dlから236 mg dlへ，6カ月齢で113 mg dlから1437 mg dlへ，9カ月齢では131 mg dlから915 mg dlへと，食餌により極端に増加した（図7）．上記6ヵ月齢の動物に対し，トリグリセリド値の性差を検討した結果，空腹時の血清トリグリセリド値には性差を認めないが，食後のレベルは雄性が1844 mg dl，雌性が675 mg dlであり，雄性の方が顕著に高いという結果を得た（図8）．

8. PHTの食後のリポタンパク分画

PHTの食後のリポタンパク分画についてアガロースゲル電気泳動法を用いて検討した結果，他のリポタンパクに対して食後のVLDLコレステロールとVLDLトリグリセリドの増加が顕著であった．VLDL (Very Low Density Lipoprotein：超低比重リポタンパク）はカイロミクロンにつぐ大きさを持つ粒子で，アガロース電気泳動ではpreβ位に分画される．VLDL

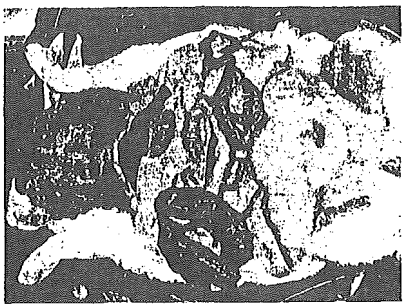

図9 PHTウサギ（雌2.5年齢）の腹腔内脂肪

の役割は肝臓で生合成されるトリグリセリドやコレステロールを，血中を介して全身の組織に運搬することにあるとされている．PHTの血中におけるVLDLの増加は，肝臓からのVLDL分泌亢進なのか，血中のVLDLの代謝が阻害されているかは不明である．また，食後に小腸上皮細胞で生成されるカイロミクロンおよびカイロミクロンレムナントの動態についても未検討である．

9. PHTの耐糖能異常と内臓脂肪

PHTにヒトの経口糖負荷試験に準じて経口糖負荷試験（1.5 g/kg OGTT）を行った結果，日本白色家兎では，負荷後2時間値が負荷前の血糖値（140 mg/dl）に戻ったのに対し，PHTでは200 mg/dl以上を示した．各血糖測定値の和である血糖和も統計学的有意な高値を示した．この結果からPHTは耐糖能異常を示すことが分かった．耐糖能異常の原因については不明である．また，同様の試験において，血中のインスリン濃度について検討した結果，PHTはインスリン抵抗性を示した．PHTの耐糖能異常ならびにインスリン抵抗性は，静脈内糖負荷試験（0.6 g/kg IVGTT）においても確認された．PHTはJWに比較すると腸管膜や腎周囲などの腹腔内に多量の脂肪組織が存在する．図9に2.5年齢雌のPHTの内臓脂肪を示した．

10. 食後高トリグリセリド血症家兎の生殖と寿命

PHTの体型は日本白色家兎と同等か若干小型であり，寿命は野生型（対照）と比較して大差を認めなかった．また，正常の生殖活動が観察された．

11. 終わりに

これまで数多くの実験的研究により，虚血性心疾患等の動脈硬化性疾患に対する高コレステロール血症の影響が解明されてきた．また最近，高トリグリセリド血症を呈するモデルウサギが開発され(9-12)，多くの研究に用いられているが，高脂肪食の負荷が伴い，PHTと比較して血清脂質値が低い．著者らが開発したPHT家兎は，通常食環境下で動脈硬化性疾患に対する血中のコレステロール高値の要因を排除し，トリグリセリドのみの影響を研究する上で有用な動物モデルと考える．さらに，虚血性心疾患を取り巻く危険因子として，高トリグリセリド血症，肥満，高血圧，耐糖能異常などが挙げられ，これら危険因子の重複が虚血性心疾患のリスクを相乗的に増加させている．個々のリスクの重症度とは別に，リスクが重複することが重要とされ，その中心に高トリグリセリド血症がある．PHT家兎は食後高トリグリセリド血症，耐糖能異常，内臓脂肪の蓄積等が確認されることから，生活習慣病やそれらの重積によって引き起こされるmultiple risk factor syndromeと虚血性心疾患との関係を解明するモデル動物として期待している．PHTウサギを用いた実験をご計画の場合は，山形大学医学部附属動物実験施設のホームページ（http://www.id.yamagata-u.ac.jp/Animal/animal.htm）をご参照頂くか，メール（tito@med.id.yamagata-u.ac.jp）にてお問い合わせください．

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Diminution of angiotensin II-induced contraction of the abdominal aorta isolated from Watanabe heritable hyperlipidemic rabbits

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Abstract

The purpose of this study was to investigate the changes in vasocontractile responses in atherosclerosis, using abdominal aortic strips isolated from Watanabe heritable hyperlipidemic (WHHL) rabbits and Japanese White (control) rabbits. The aortic strips from WHHL rabbits showed a significantly lower contractile response to angiotensin II than that in strips from control rabbits. The contractile responses to phenylephrine and 5-hydroxytryptamine were not different in WHHL and control groups. The contractile response to angiotensin II was higher in endothelium-denuded aortic strips than in endothelium-intact strips, but to a greater extent in the control group than in the WHHL group. The contractile response to angiotensin II in the absence of the endothelium was also lower in the WHHL group than in the control group. Pretreatment with N^G-nitro-L-arginine significantly increased the contractile response to angiotensin II in the endothelium-intact aortic strips in both the WHHL and control groups, while pretreatment with diclofenac did not affects the aortic contractile response to angiotensin II. The contractile responses to angiotensin II in the presence of N^G-nitro-L-arginine and diclofenac were lower in the WHHL group than in the control group. The contractile response to angiotensin II in the presence of PD123319 was also lower in the WHHL group than in the control group. Endothelium-dependent relaxation by acetylcholine occurred to the some extent in the WHHL and control groups. These results suggest that the WHHL rabbit abdominal aorta displays attenuated angiotensin II-induced contraction, mainly due to an abnormality in the angiotensin II-specific contractile pathway of the medial smooth muscle.

Key words: hyperlipidemia, atherosclerosis, vasoconstriction, angiotensin II, endothelium

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Introduction

Both dyslipidemia and hypertension are major risk factors during the progress of atherosclerosis. As a positive correlation between blood pressure and serum cholesterol level is known from epidemiological studies (Kannel, 1988), it is of interest to investigate how dyslipidemia affects arterial tone. Abnormalities in vasocontractile responses have been reported in animals with atherosclerosis induced by hypercholesterolemia. 5-Hydroxytryptamine-induced contraction is known to be increased in arteries of Watanabe heritable hyperlipidemic (WHHL) rabbits (Yokoyama *et al.*, 1983) as well as in those of rabbits with diet-induced hyperlipidemia (Henry and Yokoyama, 1980; Merkel *et al.*, 1990; Chin *et al.*, 1990). However, the reported change in angiotensin II contraction in atherosclerotic vessels is not consistent (Merkel *et al.*, 1990; Dam *et al.*, 1997; Yang *et al.*, 1998). The rabbit thoracic aorta has often been used for the study of vasocontractility in experimental atherosclerosis. On the other hand, little is known about the vascular response of the abdominal aorta isolated from WHHL rabbits. We have recently reported that angiotensin II-induced contraction was attenuated in the thoracic aorta isolated from WHHL rabbits (Shishido *et al.*, 2004). However, the detailed mechanism of this attenuation of angiotensin II contraction still remains to be determined. Angiotensin II is a major vasoactive substance involved in the pathophysiology of hypertension and atherosclerosis. In addition to having a potent vasoconstrictive action, angiotensin II induces the release of vasodilatory substances such as nitric oxide (NO) and prostacyclin, mainly from the vascular endothelium (Vane and Botting, 1993). These suppress the contractile component of the vascular response to angiotensin II. Thus, the angiotensin II-induced vasocontractile response is increased in the absence of the endothelium (Yilmaz *et al.*, 1987; Zhang *et al.*, 1994). In the present study, we investigated whether angiotensin II-induced contraction was altered in the abdominal aorta of WHHL rabbits and was modulated by vasodilatory substances released from the vessels.

Materials and Methods

Animals

The study protocols regarding treatment of animals were in accordance with the Guidelines for Experiments Using Laboratory Animals in Yamagata University School of Medicine. Male WHHL rabbits and Japanese White (control) rabbits aged 3–4 months were used in the present study. Each rabbit was housed individually in a controlled environment with unlimited access to water and was fed standard rabbit chow (120 g/day, Labo R Grower, Nihon Nosan Kogyo, Ltd., Tokyo, Japan). The animals were anesthetized with an intravenous administration of 30 mg/kg sodium pentobarbital, and segments of the abdominal aorta were carefully removed and immediately immersed in ice-cold Krebs-Henseleit solution for isometric tension study.

Tissue preparation

Excess fat and connective tissue were carefully removed from the dissected abdominal aortae. The vessels were cut into 3 mm long rings which were then cut open. In some strips,

the endothelium was removed by gentle rubbing of the intimal surface with a moistened cotton swab.

Tension measurement

Each aortic strip was suspended in an organ bath containing 10 ml of Krebs-Henseleit solution. The composition of the solution was as follows (in mM): NaCl 118, KCl 4.7, $NaHCO_3$ 24.9, $MgSO_4$ 1.18, KH_2PO_4 1.18, $CaCl_2$ 2.5, glucose 11.1, and ascorbic acid 0.057. The solution was saturated with a mixture of 95% O_2 and 5% CO_2 at 37°C (pH 7.4). The developed tension was recorded with an isometric force transducer (7T-15-240, Orientec, Tokyo, Japan). After an equilibration period of 1 hr with a resting tension of 1 g, each strip was contracted with 66.7 mM KCl repeatedly until a reproducible contraction was obtained. A solution containing a high concentration of K^+ solution was made by substituting NaCl with equimolar KCl. Contraction level was expressed as a percentage of the maximal contraction induced by the high potassium solution. Ascorbic acid (0.057 mM) did not affect angiotensin II-induced contraction of aortic strips isolated from WHHL and control rabbits. Relaxation level was expressed as a percentage of the pre-contractile tension induced by phenylephrine (100 nM). Removal of the endothelium was verified by the disappearance of relaxation induced by acetylcholine (1 μM) in strips precontracted with phenylephrine (100 nM).

Measurement of blood lipids

Blood was sampled from a marginal ear artery 18 hr after the last feed. Plasma was separated from the blood samples by centrifugation and stored at –80°C until measurement. Plasma triglyceride and total cholesterol concentrations were measured by enzymatic methods using commercial kits.

Drugs

The drugs used were as follows: angiotensin II, phenylephrine hydrochloride, 5-hydroxytryptamine creatinine sulfate, N^G-nitro-L-arginine, diclofenac and PD123319 (Sigma Chemical, St. Louis, MO, U.S.A.). Angiotensin II, phenylephrine and 5-hydroxytryptamine were dissolved in distilled water to make stock solutions of 0.1 mM, 10 mM and 10 mM, respectively, and diluted with 0.9% NaCl before use. N^G-nitro-L-arginine and diclofenac were dissolved in distilled water to make stock solutions of 10 mM and diluted with 0.9% NaCl before use. PD123319 was dissolved in dimethylsulfoxide to make a stock solution of 10 mM and diluted with 0.9% NaCl before use.

Statistics

Data was expressed as the mean ± S.E.M. Statistical analysis was done using repeated measures ANOVA (analysis of variance) followed by the Scheffé F-test. $P<0.05$ was considered to be statistically significant.

Table 1 Body weight and serum lipid levels of WHHL rabbits and control JW rabbits.

	Body weight (kg)	Serum triglyceride (mg/dl)	Serum total cholesterol (mg/dl)
WHHL rabbits	2.30 ± 0.01*	2035.9 ± 445.4*	1131.5 ± 65.5*
Control rabbits	2.53 ± 0.01	65.0 ± 18.9	53.2 ± 3.6

Data are shown as the mean ± SE. Asterisks denote significant difference from values of the control rabbits ($P<0.01$). n=17–20.

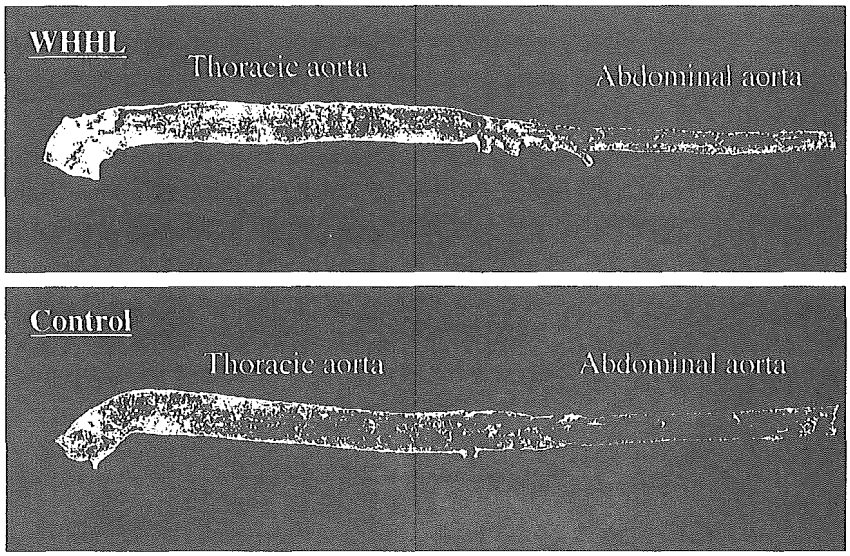

Fig. 1. Representative macroscopic images of aortae isolated from Watanabe hereditary hyperlipidemic (WHHL) and Japanese White (control) rabbits. Note the marked plaques in the thoracic region of the WHHL aorta.

Results

Body weight and blood lipid levels

Table 1 provides a comparison of the body weight and blood lipid levels of the WHHL and control rabbits. Body weight was slightly but significantly lower in the WHHL rabbits than in the control rabbits. Serum triglyceride and total cholesterol levels were markedly higher in the WHHL rabbits than in the control group.

Macroscopic observation of the aortae

Figure 1 compares representative macroscopic images of a descending aorta isolated from both a WHHL and a control rabbit. In the control rabbit aorta, no atherosclerotic plaques were observed. In the WHHL rabbit aorta, the thoracic portion of the descending aorta displayed large plaques, while plaques were not apparent in the abdominal portion of the aorta.

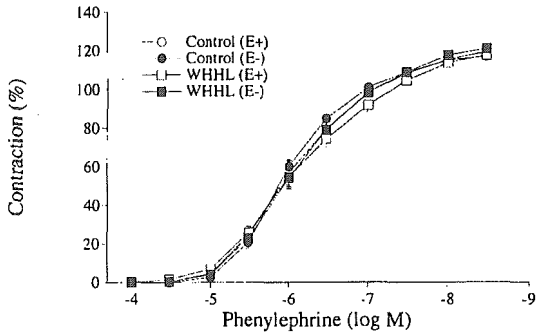

Fig. 2. Phenylephrine-induced contraction of abdominal aortic strips prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. E+, endothelium-intact; E–, endothelium-denuded. n=6–10.

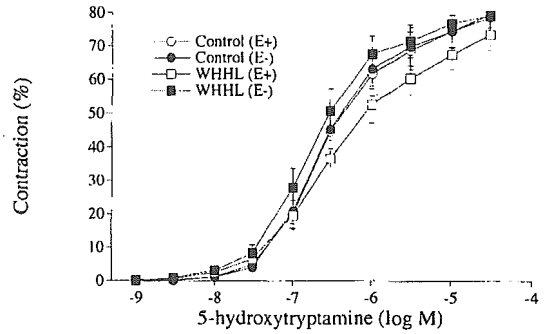

Fig. 3. 5-Hydroxytryptamine-induced contraction of abdominal aortic strips prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. E+, endothelium-intact; E–, endothelium-denuded. n=5–7.

Contractile responses to KCl, phenylephrine and 5-hydroxytryptamine

The maximum level of KCl-induced contraction of aortic strips was not significantly different between WHHL rabbits and control rabbits [8.39 ± 0.42 mN/mg tissue (WHHL) *v.s.* 8.53 ± 0.35 mN/mg tissue (control)]. Thus, we used this as the standard level of contraction for each strip. Both phenylephrine- and 5-hydroxytryptamine-induced contractile responses of aortic strips occurred to the same extent in both the WHHL and control groups (Figs. 2 and 3).

Angiotensin II-induced contractile responses in the presence or absence of endothelium

Figure 4 displays the concentration-force relationships of angiotensin II contracture of the aorta isolated from both WHHL and control rabbits in the presence or absence of endothelium. In each case, the angiotensin II-induced contraction was higher in strips without endothelium than in those with the endothelium. However, the increment in the angiotensin II-induced contraction which occurred after removal of the endothelium was less in the WHHL aorta than in the control aorta. Regardless of the presence of the endothelium, angiotensin II-induced contraction in the WHHL group was significantly lower than in the control group.

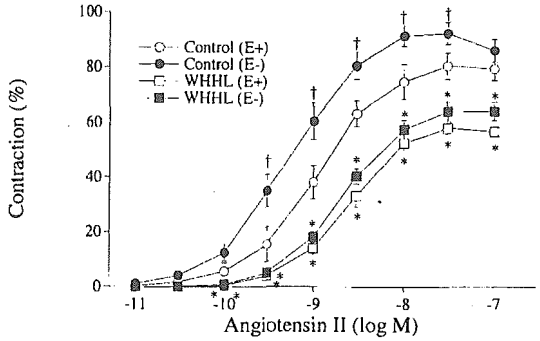

Fig. 4. Angiotensin II-induced contraction of abdominal aortic strips prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. E+, endothelium-intact; E–, endothelium-denuded. *, significantly different ($P<0.05$) from the values of strips isolated from the control rabbits; †, significantly different ($P<0.05$) from the values of strips with endothelium. n=5–12.

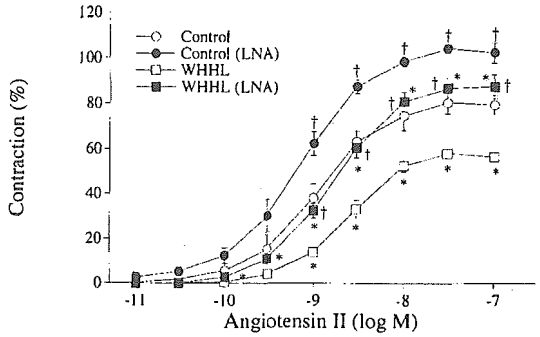

Fig. 5. Angiotensin II-induced contraction of abdominal aortic strips (endothelium-intact) prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. LNA: The aortic strips were pretreated with N^G-nitro-L-arginine (LNA, 100 μM) for 20 min before stimulation with angiotensin II. *, significantly different ($P<0.05$) from the values of strips isolated from the control rabbits; †, significantly different ($P<0.05$) from the values of strips incubated with N^G-nitro-L-arginine. n=5–12.

Angiotensin II-induced contraction in the presence of nitro-L-arginine

In both the WHHL and control groups, pretreatment with nitro-L-arginine significantly enhanced the contractile response to angiotensin II in aortic strips with intact endothelium (Fig. 5). Angiotensin II-induced contraction in the presence of nitro-L-arginine was significantly lower in the WHHL group than in the control group.

Angiotensin II-induced contraction in the presence of diclofenac

In both the WHHL and control groups, the contractile response to angiotensin II in the aorta with the intact endothelium was not significantly affected by diclofenac (Fig. 6). The angiotensin II-induced contraction in the presence of diclofenac was significantly lower in the WHHL group than in the control group.

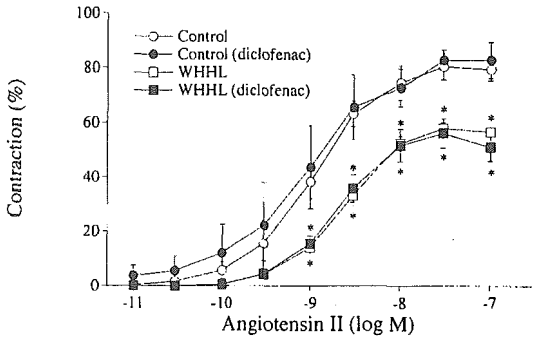

Fig. 6. Angiotensin II-induced contraction of abdominal aortic strips (endothelium-intact) prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. diclofenac: The aortic strips were pretreated with diclofenac (10 μM) for 20 min before stimulation with angiotensin II. *, significantly different (P<0.05) from the values of strips isolated from the control rabbits; †, significantly different (P<0.05) from the values of strips incubated with diclofenac. n=5–12.

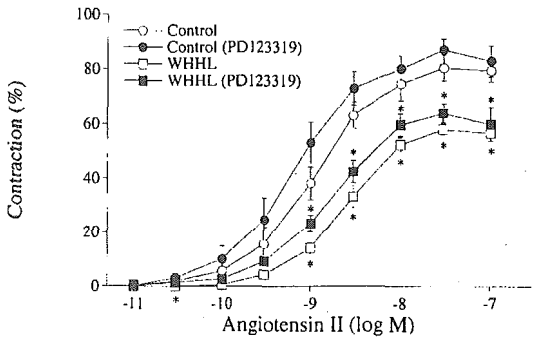

Fig. 7. Angiotensin II-induced contraction of abdominal aortic strips (endothelium-intact) prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. PD123319: The aortic strips were pretreated with PD123319 (1 μM) for 20 min before stimulation with angiotensin II. *, significantly different (P<0.05) from the values of strips isolated from the control rabbits. n=5–12.

Angiotensin II-induced contraction in the presence of PD123319

In both the WHHL and control groups, PD123319 only slightly but not significantly increased the angiotensin II-induced contraction of aortic strips with an intact endothelium (Fig. 7). The angiotensin II-induced contraction in the presence of PD123319 was significantly lower in the WHHL group than in the control group.

Acetylcholine-induced relaxing response

Acethylcholine-induced relaxation of aortic strips occurred to the same extent in both the WHHL and control groups (Fig. 8).

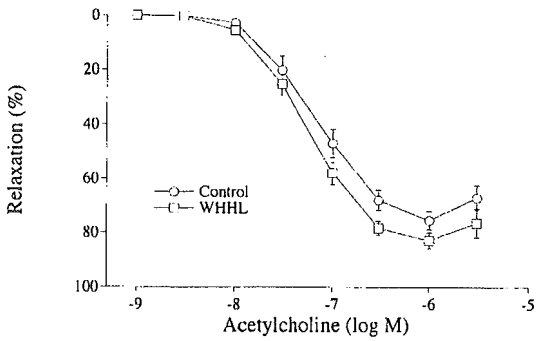

Fig. 8. Acetylcholine-induced relaxation of abdominal aortic strips (endothelium-intact) prepared from Watanabe heritable hyperlipidemic (WHHL) and control rabbits. n=6–10.

Discussion

There have been conflicting results reported about the contractile responses of atherosclerotic arteries to angiotensin II. Dam *et al.* (1997) reported that contractile responses to angiotensin II and methoxamine were decreased in the thoracic aorta but not in the iliac artery of rabbits fed with a cholesterol diet (0.3%) for 12 weeks. On the contrary, Yang *et al.* (1998) found that angiotensin II contraction, as well as its type 1 receptor expression, was increased in the thoracic aorta isolated from rabbits fed with a diet of cholesterol (1%) and coconut oil (4%) for 10 weeks. Moreover, Merkel *et al.* (1990) observed no change in angiotensin II-induced contraction of the abdominal aorta isolated from rabbits fed with a cholesterol-free, casein-rich diet for 10 weeks. These discrepant results might be due to the stage and severity of the atherosclerosis in the vessels used for the experiments. On the other hand, little is known about changes in angiotensin II contraction in arteries of WHHL rabbits. Our recent study has shown that the thoracic aorta isolated from WHHL rabbits displayed a decreased contractile response to angiotensin II, while its type 1 (AT1) receptor expression was increased in the aorta of young WHHL rabbits at 3–4 months of age (Shishido *et al.*, 2004). The present study is the first to demonstrate that angiotensin II-induced contraction was also specifically attenuated in the abdominal aorta of WHHL rabbits, while their 5-hydroxytryptamine-induced contraction was not significantly different from the aorta of control rabbits. However, both the abdominal aorta and the thoracic aorta of diet-induced atherosclerotic rabbits showed augmented contractile responses to 5-hydroxytryptamine (Henry and Yokoyama, 1980; Merkel *et al.*, 1990; Chin *et al.*, 1990). The WHHL rabbit aorta has also been reported to show increased contractility in response to 5-hydroxytryptamine (Yokoyama *et al.*, 1983). The discrepant results of the 5-hydroxytryptamine contraction in the atherosclerotic aorta in these previous studies and those of the present study may also be due to the stage and severity of atherosclerosis, because atherosclerotic plaques were not macroscopically observed in the descending aortae used in the present study. Moreover, the aorta from 1-month-old animals was reportedly more sensitive to 5-hydroxytryptamine than that from 6-month-old

animals (Wines *et al.*, 1989), suggesting that age could also affect the contractile response to 5-hydroxytryptamine.

In the present study, we examined whether endogenous vasodilatory substances were involved in attenuation of the contractile response to angiotensin II in the WHHL rabbit aorta. The decrease in contractile force produced by angiotensin II in the WHHL aorta was also observed in the absence of the endothelium, indicating that the decrease in angiotensin II contraction was not due to vasoactive substances released from the endothelium. NO and prostacyclin are major vasodilatory substances that regulate vascular tone and that are produced in both the vascular intima and media (Moncada *et al.*, 1991 Vane and Botting, 1993; Mitchell and Evans, 1998). Pretreatment of nitro-L-arginine, a NO synthase inhibitor, significantly augmented the contractile response to angiotensin II in the aorta with an intact endothelium in both WHHL and control rabbits. Thus, NO negatively regulates the contractile response to angiotensin II. However, angiotensin II-induced contraction in the presence of nitro-L-arginine was also lower in the WHHL group than in the control group, suggesting that NO is not involved in the diminution of angiotensin II contraction in the WHHL rabbit abdominal aorta. Moreover, acetylcholine-induced endothelium-dependent aortic relaxation, which is mainly due to NO release from the endothelium, was not significantly different between the WHHL and control groups. In contrast, several studies have shown a decrease in endothelium-dependent relaxation in the aorta of WHHL rabbits (Ragazzi *et al.*, 1989; Kolodgie *et al.*, 1990) as well as in the aorta of high-cholesterol-diet-induced atherosclerotic rabbits (Sreeharan *et al.*, 1986; Jayakody *et al.*, 1988). The lack of impairment of endothelium-dependent vasodilation in the WHHL rabbit abdominal aorta in the present study may also be due to a lesser degree of atherosclerosis. Moreover, our recent study demonstrated that attenuation of endothelium-dependent relaxation of the WHHL rabbit thoracic aorta, which displayed markedly large atherosclerotic plaques, was observed only in those animals with hypertriglyceridemia (Shishido *et al.*, 2004), suggesting that hypertriglyceridemia aggravates hypercholesterolemia-induced functional impairment of endothelial cells. The contractile force of the aorta in response to angiotensin II was not affected by pretreatment with diclofenac, a cyclooxygenase inhibitor, while that in the presence of diclofenac was also lower in the WHHL group than in the control group. This finding agrees with the finding of a study by Forstermann *et al.* (1984) that prostaglandin release was enhanced by angiotensin II in both the rabbit coeliac artery and pulmonary artery but not in either the aorta or femoral artery. Therefore, cyclooxygenase products such as prostacyclin do not influence the vascular tone of the angiotensin II response and are not involved in attenuation of the angiotensin II contraction of the WHHL rabbit aorta. Angiotensin II contraction is mediated mainly via stimulation of AT1 receptors in vascular smooth muscle cells. In addition, angiotensin II has recently been reported to induce vasodilation directly via stimulation of its type 2 (AT2) receptor (Widdop *et al.*, 2003). However, hypocontractility to angiotensin II of the WHHL rabbit aorta was also observed in the presence of PD123319, an AT2 receptor antagonist, indicating that AT2 receptor-mediated vasodilation is not involved in the hypocontractility of the WHHL rabbit aorta. The above findings suggest that the decrease in the angiotensin II contraction in the WHHL rabbit aorta is mainly due to an abnormality in smooth muscle contractility, and is not due to changes in the release of vasodilatory substances from the vessel wall. AT1 receptor

expression has rather been shown to be up-regulated in the thoracic aorta of WHHL rabbits (Shishido *et al.*, 2004). Thus, further study including AT1 receptor coupling with downstream signals in vascular smooth muscle cells is needed to clarify the mechanism involved in the attenuated contractile response to angiotensin II in the WHHL aorta. Furthermore, it is also of interest to determine whether other angiotensin II-mediated biological actions in vascular smooth muscle, such as protein synthesis, mitogenesis and hypertrophy, are altered in arteries of WHHL rabbits.

In conclusion, the WHHL rabbit abdominal aorta displays attenuated angiotensin II-induced contraction, which is suggested to be mainly due to an abnormality in the AT1-receptor-mediated contractile pathway of medial smooth muscle.

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PROGRESSION OF SEVERE ATHEROSCLEROSIS AND INCREASED ARTERIAL PULSE PRESSURE IN THE NEWLY DEVELOPED HERITABLE MIXED HYPERLIPIDAEMIC RABBITS

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SUMMARY

1. We have recently segregated a new line of rabbit, named TGH, with severely high levels of plasma triglyceride and cholesterol. The aim of the present study was to investigate the progression of atherosclerosis and haemodynamic parameters in TGH rabbits.

2. Japanese white (JW) and TGH rabbits (24–27 months old) were anaesthetized with ketamine and xylazine. Plasma concentrations of triglyceride were 63.1 ± 8.0 and 446.0 ± 35.2 mg/dL in JW and TGH rabbits, respectively. Blood pressure was measured by a catheter implanted in the femoral artery. Histological examinations were performed using haematoxylin–eosin and elastica–Masson trichrome staining to detect atherosclerotic lesions.

3. The JW rabbits had no atherosclerotic lesions. In TGH rabbits, severe atherosclerotic lesions were observed throughout the aorta, especially in the aortic arch. Basal femoral arterial pressure was not significantly different between JW and TGH rabbits. However, the basal pulse pressure in TGH rabbits (48.3 ± 4.5 mmHg) was significantly greater than that of JW rabbits (28.0 ± 5.6 mmHg). Intravenous infusion of N^G-nitro-L-arginine methyl ester (L-NAME; 26.9 mg/kg) increased the blood pressure of TGH and JW rabbits. There was no significant difference in the response to L-NAME between the two rabbit strains.

4. The present study shows that severe atherosclerotic changes develop in TGH rabbits and suggests that the hyperlipidaemia combined with hypercholesterolaemia and hypertriglyceridaemia is an important factor for promoting atherosclerosis in TGH rabbits. The greater pulse pressure in TGH rabbits may be due to the increased vascular stiffness with atherosclerosis.

5. This newly developed TGH rabbit line of heritable hypertriglyceridaemia with hypercholesterolaemia will become a useful animal model for studies on the role of hyperlipidaemia in the progression of atherosclerosis and in many atherosclerosis-related diseases.

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INTRODUCTION

Atherosclerosis is one of the major triggers for ischaemic heart disease, cerebrovascular disease and obstructive arteriosclerosis. Various factors are involved in the progress of atherosclerosis. Of them, hyperlipidaemia, such as hypercholesterolaemia, is regarded as the most widely demonstrated risk factor for these diseases through the progression of atherosclerotic plaques based on epidemiological studies,[1] as well as experimental studies using Watanabe heritable hyperlipidaemic (WHHL) rabbits, which have a defect in their low-density lipoprotein (LDL) receptors. The WHHL rabbits have been used to demonstrate that hypercholesterolaemia promotes fatty streak formation and atherosclerosis at the vascular wall and reduces endothelium-dependent relaxation.[2,3]

Hypercholesterolaemia may cause a progression of the formation of fatty streaks, fibrous plaques and atherosclerosis via uptake of oxidized LDL into foam cells.[4,5] Because cholesterol is metabolised and finally transported into blood as LDL, cholesterol is regarded as the most important factor for the promotion of early stage atherosclerosis. In addition to pathohistological changes, hyperlipidaemia can induce dysfunction of blood vessels. This may be due to endothelial injury caused by lipoproteins, such as oxidized LDL and β very low-density lipoprotein (VLDL).[6] The production of nitric oxide (NO) by activation of endothelial NO synthase (eNOS) is diminished and the endothelium-dependent vasorelaxant responses are decreased in atherosclerosis and hyperlipidaemic conditions.[6,7]

In contrast with the important role of cholesterol, it has not been evaluated, in detail, whether hypertriglyceridaemia is a risk factor for the progression of atherosclerosis. Recent epidemiological studies have demonstrated that hypertriglyceridaemia is a risk factor for coronary heart diseases.[8–10] The remnant lipoproteins that contain triglyceride are hydrolysed by lipoprotein lipase and are able to infiltrate into vessel walls to develop atherosclerotic lesions.[11] Macrophages can take up remnant lipoproteins via surface receptors (LDL receptor, VLDL receptor and apoB48 receptor) to become foam cells. As a result, endothelial function may be impaired, smooth muscle cell proliferation is enhanced and the vascular contractile response to various agonists is enhanced.[5] However, in physiological studies on vascular function, the contribution of hypertriglyceridaemia

to endothelial and medial smooth muscle function has not been determined clearly.[12–14]

We have recently segregated a new rabbit line, namely TGH rabbits, of mixed hyperlipidaemia with markedly high concentrations of plasma triglycerides (TG; > 500 mg/dL in young) and cholesterol[15,16] to investigate the role of mixed hyperlipidaemia in the progression of atherosclerosis. Until now, the haemodynamic parameters of TGH rabbits have not been determined. The aim of the present study was to examine changes in the haemodynamic parameters in TGH rabbits and to evaluate the role of mixed hyperlipidaemia in the progression of atherosclerosis.

METHODS

Animals

Experiments were performed in accordance with the *Guide for Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85–23, revised 1996; http://www.nap.edu/readingroom/books/labrats/index.html) and under the regulations of the Animal Care Committee of Yamagata University School of Medicine.

Watanabe heritable hyperlipidaemic rabbits were bred since 1991 in the Laboratory Animal Center, Yamagata University School of Medicine. There was wide interindividual variability in plasma TG levels in WHHL rabbits. The definition of hypertriglyceridaemia in the present study was TG > 500 mg/dL in plasma. Based on the level of plasma TG (> 500 mg/dL) of WHHL rabbits, selected inbreedings were repeated up to the seventh generation. The result was that hypertriglyceridaemia had a high penetrance with 93% and finally 100% of phenotype expression at the fourth and fifth generations, respectively. Therefore, these rabbits were named 'TGH rabbits'. We used the seventh generation of TGH rabbits in the present study.

Male 24–27-month-old Japanese white rabbits (weighing 3.7 ± 0.1 kg; $n = 5$) and TGH rabbits (weighing 2.50 ± 0.02 kg; $n = 6$) were used to measuring haemodynamic parameters and for histological examination. In addition, three male young (3 months) and aged (30 months) TGH rabbits were used for histological examination to investigate the progression of atherosclerosis. All animals were housed individually in a controlled environment with unlimited access to water and were fed standard rabbit chow (120 g/day; Labo R Grower; Nihon Nosan Kogyo, Tokyo, Japan). Rabbits were anaesthetized with ketamine hydrochloride (35 mg/kg) and xylazine (5 mg/kg) via the marginal ear vein. Then, anaesthesia was maintained by continuous intravenous infusion of ketamine (0.11 mg/kg per min) and xylazine (0.02 mg/kg per min). Maintenance of body temperature was achieved with the aid of a heating pad.

Measurements of arterial blood pressure and electrocardiogram

Catheters were placed in the right femoral artery for continuous recording of arterial blood pressure and in the left femoral vein for the infusion of drugs. Arterial pressure was measured by a pressure transducer and recorded on a thermal array recorder (RTA 1200M; Nihon Kohden, Tokyo, Japan). Electrodes were attached to the shaved area on each limb for recording the surface electrocardiogram (ECG). The ECG was recorded continuously using limb lead II. In some experiments, the NOS inhibitor N^G-nitro-L-arginine methylester (L-NAME) was infused through the left femoral vein. We prepared a 100 mmol/L stock solution of L-NAME (26.9 mg/mL) and used 26.9 mg/kg L-NAME to inhibit NOS sufficiently and specifically.[17–19]

Histological analysis of aorta

Rabbits were killed with an intravenous overdose of pentobarbitone sodium (300 mg) at the end of each experiment. Thoracic aortas obtained from JW and TGH rabbits were divided into four portions (I, aortic arch; II, proximal thoracic aorta; III, middle thoracic aorta; and IV, distal thoracic aorta) and fixed overnight in 10% formaldehyde at 4°C. Tissues were embedded in paraffin and cut into 4 μm cross-sections. Microscopic examination of haematoxylin–eosin- and elastica–Masson's trichrome-stained sections was performed to assess atherosclerotic changes in the aortas. In the stained cross-sections, the luminal surface and atherosclerotic plaque areas were compared among aortic portions.

Plasma lipid analysis

Blood samples were taken from a marginal ear artery 18 h after the last feeding. All blood samples were centrifuged at 1 000 *g* for 15 min at 4°C and the plasma was stored at –80°C until assay. Plasma concentrations of total cholesterol and TG were measured by enzymatic methods using SPOTCHEM-EZ (Arkray, Kyoto, Japan).

Statistical analysis

All data are expressed as the mean±SEM. Statistical analysis was performed with unpaired *t*-tests or Welch's *t*-tests. To compare changes in blood pressure, non-parametric analysis (Mann–Whitney *U*-test) was used. $P < 0.05$ was considered statistically significant.

Drugs

Ketamine hydrochloride (Sankyo Pharmaceutical, Tokyo, Japan), xylazine (Bayer, Tokyo, Japan) and L-NAME (Sigma Chemical, St Louis, MO, USA) were used.

RESULTS

Bodyweight and plasma cholesterol and TG levels

The bodyweight of TGH rabbits was significantly lower than that of JW rabbits. Total cholesterol levels in TGH rabbits were significantly higher than those in JW rabbits. In addition, plasma TG levels of TGH rabbits were markedly higher compared with those of JW rabbits. Plasma cholesterol concentrations were 58.2 ± 5.1 and 442.7 ± 27.7 mg/dL in JW and TGH rabbits, respectively. Concentrations of TG were 63.1 ± 8.0 and 446.0 ± 35.2 mg/dL in JW and TGH rabbits, respectively (Table 1).

Histological study

Figure 1 shows the histological changes in each portion of the aortas of JW and TGH rabbits. In JW rabbits, no detectable atheromatous lesions were observed in any portion of the aorta. Examination revealed normal intima containing an intact monolayer of endothelium and a major portion of the media. In contrast, remarkable atherosclerotic

Table 1 Plasma lipid concentrations in adult Japanese white and TGH rabbits

	Bodyweight (kg)	Plasma lipid (mg/dL)		Age (months)	*n*
		Total cholesterol	Triglyceride		
JW	3.73 ± 0.10	58.2 ± 5.1	63.1 ± 8.0	24.8 ± 0.4	5
TGH	2.47 ± 0.02	442.7 ± 27.7	446.0 ± 35.2	26.7 ± 0.3	6

JW, Japanese white rabbits; TGH, high-triglyceride rabbits.