一般住民における不整脈ならびに
心血管疾患にかかわる炎症性バイオマーカーの現状の実態調査

岡山大学大学医歯薬学総合研究科
循環器内科学　中村　一文・松三　博明・上岡　亮・三好　亨・伊藤　浩
公衆衛生学　　江口　依里・汪　達紘・荻野　景規
井原市民病院　　内科　山田　信行・斎藤　大治

研究の背景

高齢化に伴い動脈硬化症・心血管疾患は増加している。一般住民におけるそのリスクの現状を把握するため疫学調査が必要である。

心房細動などの不整脈に伴う脳梗塞は患者の生活の質(QOL)ならびに予後を著しく悪くし, 脳梗塞発症の予防をしていくことが極めて重要である。不整脈にて病院に通院している人は, 適切な抗凝固剤の投与により, その予防が可能である。しかしながら, 1)無症候性の心房細動・不整脈患者, 2)不整脈の診断を下されているのに病院に行っていない人, 3)不整脈の自覚があるのに病院に行っていない人はリスクが高い。

また近年, 動脈硬化症が, 肥満, 高血圧, 高血糖等により誘発される低レベル持続性炎症として認知されている。魚油中の n-3 系多価不飽和脂肪酸であるエイコサペンタエン酸（EPA）には抗炎症作用があり、血液中 EPA 濃度ならびに EPA と n-6 系多価不飽和脂肪酸であるアラキドン酸(AA)比は動脈硬化の予防につながるバイオマーカーと考えられている。

研究の目的

本研究では岡山県井原市における特定健康診査（40 歳から 74 歳）及び 75 歳以上の健康診査の受診者を対象に, 下記の 2 つの検討を行った。

研究①　不整脈患者の割合の現状調査: 1)無症候性の心房細動・不整脈患者, 2)不整脈の診断を下されているのに病院に行っていない人, 3)不整脈の自覚があるのに病院に行っていない人の割合をアンケート並びに心電図にて検討する。

研究②　抗炎症性バイオマーカーの EPA 値推定につながる n-3 系多価不飽和脂肪酸の摂取量調査：食事の内容をアンケート調査することにより、n-3 系多価不飽和脂肪酸の摂取量を推定する。

研究の内容，方法

（1）　同意を得られた者に不整脈の自覚症状・既往歴・通院歴, アレルギー性疾患の既往歴及び生活習慣等に関するアンケート (井原市民生活習慣調査票) を記入して頂く。

（2）　健診の必修項目（心電図と採血）を通常どおり受けて頂く。

（3）同意を得られた方の、心電図所見を収集する。

（4）データ解析は，統計ソフト SPSS を用いて行う。

（5）本研究は岡山大学大学院医歯薬学総合研究科の倫理委員会の承認をえている（受付番号 547)。

各研究の詳細

研究①不整脈患者の割合の現状調査題

心房細動患者の医療機関を受診している割合い

－井原市健診受診者の調査研究（IBARA-AF 研究)－

研究の概要

【背景】心房細動（AF）があるにもかかわらず、医療機関を受診していない人を

発見・加療していくことは、脳塞栓症の予防につながると考えられるが、その割合いは不明である。岡山県井原市において、健診にて心電図上 AF を認める患者の医療機関への受診の割合いを調べた。
【方法と結果】
40 歳以上７４歳未満の特定健康診査(1082 例)と 75 歳以上の後期高齢者健康診査(547 例) をうけた 1629 例の内、同意をえられた 1070 例 (特定 522 例と後期高齢者 547 例)を対照に調査した。心電図上 AF を認めた割合いは特定 7 例(1.3%)・後期高齢者 17 例(3.1%)： 総 24 例 (2.3%)であった。そのうち無症候は 3 例 (3/24 = 12.5%)であった。不整脈で定期的に医療機関を受診している者 (受診中 AF)・発作時のみ受診する者 (発作時受診 AF)・過去に受診したことあるが現在は通院していない者 (非受診 AF)・受診したことがない者 (未受診 AF)を調査した。受診中 AF 18 例(75%)・発作時受診 AF 1 例(4%)・非受診 AF 2 例(8%)・未受診 AF 3 例 (13%)であった。
【結語】今回の検討では AF 患者のうち約１／４の例が定期受診をうけていなかった。AF の危険性の一般への啓蒙が今後重要である。

背景と目的

- 医療機関を受診していない心房細動患者を発見し受診を促す事は，脳梗塞の発症予防につながると考えられる。
- 過去の疫学調査から本邦における心房細動の有病率が報告されているが，その受診状況について調査された大規模研究はこれまでに報告が無い。
- 岡山県井原市の健康診断にて実施された心電図で，心房細動と診断された患者の医療機関への受診状況を調査した。
- 本研究の井原市は県の西南部に位置し、ほとんどが山々に囲まれた農山村です。人口は約 44000 人で、日本全体の割合と比較し、比較的高齢者の多い地域である。

対象と方法

- 【対象】 岡山県井原市における，平成 24 年度の特定健康診査（40 歳～74 歳）および後期高齢者健康診査（75 歳以上）の受診者。
- 【方法】 健診で実施された心電図における心房細動患者の頻度を調べ，自覚症状の有無と医療機関への受診状況について，アンケートによる調査を実施した．図 1 ならびに 2 に調査票を示す。

不整脈で病院にかかったことがありますか？もしくは病院にかかっていますか？
下記の中から 選んで(　)に○を記入ください。
①(　) はい、現在定期的に通院しています。
→通院中
②(　) はい、発作がおきたときだけ病院にいきます。
→発作時受診
③(　) はい、以前かかったことがありますが、現在は通院しておりません。
→非受診 (過去に受診)
④(　) いいえ、かかったことはありません。
→未受診

図 1 井原市民生活習慣調査票 1

1) 動悸を感じることがありますか？
1. はい　2. いいえ
2) 脈の乱れ(脈が不規則または飛ぶこと)がありますか？
1. はい　2. いいえ　3. わからない
3) 胸痛がありますか？
1. はい　2. いいえ
4) 胸苦しさがありますか？
1. はい　2. いいえ
5) 全身がだるい感じがありますか？
1. はい　2. いいえ
6) めまいがありますか？
1. はい　2. いいえ

図 2 井原市民生活習慣調査票 2

結果

- 【受診者数と調査数】

40 歳以上７４歳未満の特定健康診査(1082 例)と 75 歳以上の後期高齢者健康診査(547 例) をうけた 1629 例の内、同意をえられた 1070 例を対照にアンケート調査した(図 3)。

図 3　健康診断実施数とアンケート調査数

	保険		計
	国保	後期高齢	
実施人数	1082	547	1629
アンケート協力数 (%)	523 (48%)	547 (100%)	1070 (66%)

- 【心電図上の心房細動患者の頻度】

心電図上 AF を認めた割合いは特定 7 例(1.3%)・後期高齢者 17 例(3.1%)： 総 24

例（2.3%）であった（図4）。

	保険		計
	国保	後期高齢	
協力数	523	547	1070
心電図上 心房細動患者 (%)	7 (1.3)	17 (3.1)	24 (2.2)

図4 心電図上の心房細動患者の頻度

- 【心電図上の心房細動患者の受診状況】

不整脈で定期的に医療機関を受診している者（受診中 AF）・発作時のみ受診する者（発作時受診 AF）・過去に受診したことあるが現在は通院していない者（非受診 AF）・受診したことがない者（未受診 AF）を調査した。受診中 AF 18 例(75%)・発作時受診 AF 1 例(4%)・非受診 AF 2 例(8%)・未受診 AF 3 例（13%)であった（図5)。心房細動患者の4人に1人が医療機関を定期受診していない事が分かった。

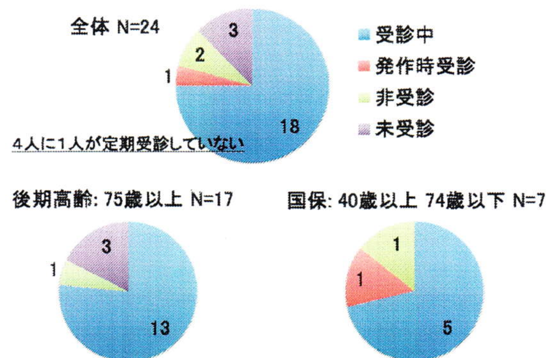

図5 心電図上の心房細動患者の受診状況

- 【全ての心房細動患者の受診状況】

アンケートで心房細動の既往ありとされた患者を含めた全ての患者の受診状況を示す。同様に心房細動患者の約4人に1人が医療機関を定期受診していない事が分かった(図6)。

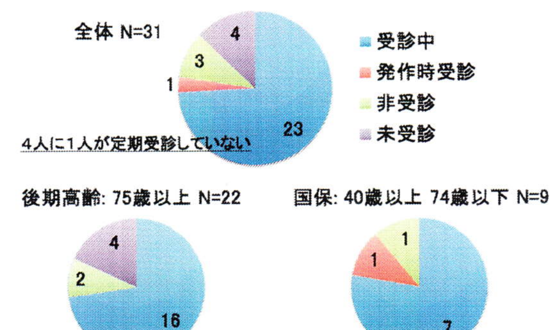

図6 全ての心房細動患者の受診状況

- 【心房細動患者の $CHADS_2$ score】

医療機関を受診していない心房細動患者 10 人中 9 人が，$CHADS_2$ score 1 点以上であった(図7)。

国保: 40歳以上 74歳以下

No.	年齢	性別	心電図所見	心房細動既往	自覚症状	CHADS2	受診状況
1	60	男	心房細動	なし	なし	0	発作時受診
2	74	男	心房細動	なし	なし	1	非受診

後期高齢: 75歳以上

No.	年齢	性別	心電図所見	心房細動既往	自覚症状	CHADS2	受診状況
3	79	女	正常	あり	あり	1	非受診
4	79	女	心房細動	なし	あり	1	非受診
5	86	男	心房細動	なし	あり	2	未受診
6	81	女	心房細動	なし	なし	2	未受診
7	77	女	不完全右脚ブロック	あり	あり	2	未受診
8	80	男	心房細動	あり	あり	2	未受診

図7 心房細動患者の **CHADS2 score**

結語

- 心電図で心房細動と診断できる患者の4人に1人は医療機関に定期受診していなかった。
- 健診の結果を元に受診を促す事で，脳梗塞発症の予防が期待できると考えられる。

（本研究は第30回日本心電学会学術集会にて発表した　参考文献1）(英文論文投稿準備中)。

研究②　抗炎症性バイオマーカーの EPA 値推定につながる n-3 系多価不飽和脂肪酸の摂取量調査

背景と目的

食事の内容をアンケート調査することにより、n-3 系多価不飽和脂肪酸の摂取量を推定する。

方法

- 【対象】　岡山県井原市における，平成 24 年度の特定健康診査（40 歳～74 歳）および後期高齢者健康診査（75 歳以上）の受診者。
- 【方法】　アンケートによる調査を実施した。図 8 に調査票を示す。

図 8　食事調査票

結果

- 【調査数】　1024 名分のアンケートを解析した。
- 【栄養素摂取状況】
  n-3 系多価不飽和脂肪酸を含む 1 日の栄養素の摂取量を図 9 に示す。
  摂取量の目安である 2 g/日より少なかった。

図 9　1 日の栄養素摂取状況

	男性	女性
総エネルギー (kcal)	1600 ± 387	1568 ± 356
脂肪エネルギー比率	22.9 ± 5.5	23.6 ± 4.6
蛋白質エネルギー比率	14.3 ± 2.6	14.9 ± 2.2
炭水化物エネルギー比率	62.4 ± 8.0	61.5 ± 5.8
カルシウム (mg)	470 ± 206	560 ± 195
鉄 (mg)	5.7 ± 2.0	6.3 ± 1.9
ビタミン A (μg)	457 ± 204	532 ± 197
ビタミン B1 (mg)	0.68 ± 0.23	0.75 ± 0.22
ビタミン B2 (mg)	0.85 ± 0.31	0.94 ± 0.30
ビタミン B6 (mg)	0.87 ± 0.31	0.97 ± 0.29
ビタミン B12	6.1 ± 3.2	6.8 ± 3.0
葉酸 (μg)	228 ± 90	263 ± 89
ビタミン C (mg)	79 ± 39	96 ± 39
ビタミン D (mg)	7.3 ± 4.0	8.3 ± 4.0
ビタミン E (mg)	5.1 ± 2.0	5.5 ± 1.8
ビタミン K (μg)	161 ± 79	137 ± 78
コレステロール (mg)	261 ± 109	273 ± 106
n3 系多価不飽和脂肪酸 (g)	1.5 ± 0.7	1.6 ± 0.6
n6 系多価不飽和脂肪酸 (g)	5.9 ± 2.5	6.2 ± 2.4
n－6 系脂肪酸/n－3 系脂肪酸	4.2 ± 1.5	4.0 ± 1.0
食物繊維 (g)	10.9 ± 3.9	12.3 ± 3.9

* 平均値±標準偏差

結語

n-3 系多価不飽和脂肪酸を含む 1 日の栄養素の摂取量は、目安である 2 g/日より少なかった。今後魚摂取の啓蒙活動が必要と考えられる。

(本研究は 2013.4.9 井原市民病院でおこなわれた研究結果報告会にて発表された)

追加研究

研究③ **klotho** 変異マウスにおける **EPA** による動脈石灰化の抑制

概要

背景と目的

上述の如く **EPA** は抗炎症作用と抗動脈硬化作用をもつと考えられている。

EPA 投与にて **klotho** 変異マウス において動脈石灰化が抑制できるか検討した。

方法

kloltho 変異マウスと野生型マウスにて **EPA** 投与前と投与 **4** 週間後で **CT** にて石灰化を定量する

結果

EPA 投与は **kltho** 変異マウスにおいて有意に石灰化を抑制した。さらに **EPA** の代謝物で抗炎症作用のある血中レゾルビ

ンを有意に上昇させ、酸化ストレス発生につながる **NADPH oxidase** の活性を抑制した。

結語

EPA は **klotho** 変異マウスの動脈石灰化を抑制した。今後ヒトにおいても同様に石灰化抑制作用があるか検討が必要である。

（本研究は米国心臓学会 Scientific Sessions 2014 にて発表された。さらに PLoS One 誌に論文受理された:参考文献 2）

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29 CHA_2DS_2-VASc score = 0の非弁膜症性心房細動症例に対する新規抗凝固薬使用に関する検討

兵庫県立姫路循環器病センター循環器内科
岡嶋克則 嶋根 章 木内邦彦 横井公宣 寺西 仁
千村美里 青木恒介 谷口泰代 山田慎一郎 小林征一
大西哲存 月城泰栄 澤田隆弘 三好直貴 大石醒悟
鳥羽敬義 宮田大嗣 津端英雄 松岡裕樹 川合宏哉
矢坂義則

【背景】心房細動(AF)に対してはCHA_2DS_2-VASc score≧1から新規抗凝固薬の使用が推奨されているが，除細動やカテーテルアブレーション(CA)を考慮する際にはCHA_2DS_2-VASc score = 0でも抗凝固薬の開始を検討せざるを得ない．【目的】CHA_2DS_2-VASc score = 0のAF症例に対する新規抗凝固薬の効果と安全性について検討すること．【方法】CHA_2DS_2-VASc score = 0のAFに対してダビガトランを開始した8症例(年齢49±8歳)の臨床的特徴と経過について検討した．【結果】6例はlone AF(持続性2例)，2例は心筋症(持続性1例)を有していた．全例で腎機能は正常(Ccr 110±14 ml/min)で，投与量は220mg/日が6例，300mg/日が2例と低用量が主体であり，投与後のAPTT = 43±6秒であった．平均投与期間は12±6ヵ月だったが，CAを施行した5例中4例では再発なく3〜8ヵ月後にダビガトランの中止が可能であった．全例で血栓塞栓症及び出血事象は認めなかった．【結語】CHA_2DS_2-VASc score = 0の心房細動に対する低用量を主体としたダビガトラン投与は安全であった．

30 ダビガトランを用いた心房細動アブレーション周術期における抗凝固療法の有用性と安全性

桜橋渡辺病院循環器内科
田中宣暁 井上耕一 田中耕史 豊島優子 岡 崇史
外海洋平 野里陽一 岩倉克臣 藤井謙司

【背景】心房細動(AF)アブレーションの周術期における抗凝固療法として，ワルファリンの継続投与が合併症の回避に有用であるが，出血性合併症の重症化が懸念される．一方，ダビガトランは効果の発現・消失が速やかで，周術期の休薬が短くすむ利点がある．今回，AFアブレーション周術期におけるダビガトラン使用の安全性，有効性を後ろ向きに検討した．【方法】対象は初回AFアブレーションの周術期にダビガトランを内服した連続99例．ダビガトラン内服は，アブレーション前日夕より中止，当日夕より再開とした．【結果】対象は年齢59.1±10歳，発作性AF60例(60%)．術前からのダビガトラン内服(A群)は70例，術後からの内服(B群)は29例であった．$CHADS_2$(CHA_2DS_2-VASc)スコアの平均は0.78±0.93(1.4±1.3)．内服が中断されたのは2例のみであった．術後1日目，A群で1例にTIA(右口角下垂，右上肢痺れ)を発症したが，1時間で消失した．その他の明らかな血栓塞栓症，出血性合併症を認めなかった．【結論】ダビガトランによるAFアブレーション周術期の抗凝固療法は，ワルファリン継続内服以外のもうひとつの選択肢となりうる．

参考文献 1

31 心房細動患者の医療機関を受診している割合い ―井原市健診受診者の調査研究(IBARA-AF研究)―

[1]岡山大学大学院医歯薬学総合研究科先端循環器治療学
[2]岡山大学大学院医歯薬学総合研究科公衆衛生学
[3]井原市民病院循環器内科
上岡 亮[1] 中村一文[1] 三好 亨[1] 寒川睦子[1] 麻植浩樹[1]
森田 宏[1] 汪 達紘[2] 荻野景規[2] 斎藤大治[3] 山田信行[3]
河野晋久[1] 大野祐子[1] 伊藤 浩[1]

【背景】心房細動(AF)があるにもかかわらず，医療機関を受診していない人を発見・加療していくことは，脳塞栓症の予防につながると考えられるが，その割合いは不明である．岡山県井原市において，健診にて心電図上AFを認める患者の医療機関への受診の割合いを調べた．【方法と結果】40歳以上74歳未満の特定健康診査(1,082例)と75歳以上の後期高齢者健康診査(547例)をうけた1,629例のうち，同意を得られた1,070例(特定522例と後期高齢者547例)を対照に調査した．心電図上AFを認めた割合いは特定7例(1.3%)・後期高齢者17例(3.1%)：総24例(2.3%)であった．そのうち，無症候は3例(3/24 = 12.5%)であった．不整脈で定期的に医療機関を受診しているもの(受診中AF)・発作時のみ受診するもの(発作時受診AF)・過去に受診したことあるが現在は通院していないもの(非受診AF)・受診したことがないもの(未受診AF)を調査した．受診中AF 18例(75%)・発作時受診AF 1例(4%)・非受診AF 2例(8%)・未受診AF 3例(13%)であった．【結語】今回の検討では，AF患者のうち約1／4の例が定期受診を受けていなかった．AFの危険性の一般への啓蒙が今後重要である．

32 栃木県の実地医家による心房細動患者診療の現況

[1]自治医科大学大宮医療センター循環器科
[2]獨協医科大学循環器内科
三橋武司[1] 堀中繁夫[2]

【背景】心房細動診療に関して従来の報告は大病院からのものが多かった．しかし，心房細動の母集団は大きく，その診療の大部分は開業医あるいは地域病院で行われている．さらに，新規抗凝固薬の登場で今後その診療も大きく変化してきていることが予想される．このような状況のなか，実地臨床現場の現況を調査する必要があると思われた．【対象および方法】栃木県下の開業医あるいは大学病院を除く病院勤務医に対して，2013年3〜4月に心房細動患者の外来診療状況をアンケート調査した．診療患者数は666名，診療医師は66施設の71名であった．【結果】①平均年齢は74.8歳で，75歳以上が半数以上を占めた．②加齢に伴い患者数が増加し，80歳以下では男性の方が多かったが，80歳以上では女性が逆転し，全年齢分布のなかでも最も多かった．③ワルファリンは51%に，その他新規抗凝固薬は23%に処方されており，未処方は10%であった．【結論】栃木県実地臨床の現場では，80歳以上の高齢女性が多く含まれていた．理由は不明であるが，今後詳細な調査が必要と思われた．従来の報告より抗凝固薬の処方率が高く，医師の意識の高まりと新規抗凝固薬の登場がその結果に影響を与えた可能性がある．

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参考文献　2

Eicosapentaenoic Acid Prevents Arterial Calcification in *klotho* Mutant Mice

Kazufumi Nakamura[1,*], Daiji Miura[1,2], Yukihiro Saito[1], Kei Yunoki[1], Yasushi Koyama[3], Minoru Satoh[4], Megumi Kondo[1], Kazuhiro Osawa[1], Omer F. Hatipoglu[1], Toru Miyoshi[1], Masashi Yoshida[1], Hiroshi Morita[1,5], Hiroshi Ito[1].

[1]Department of Cardiovascular Medicine, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan.

[2]Department of Basic Medicine, Nagano College of Nursing, Komagane, Japan.

[3]Department of Cardiology, Sakurabashi-Watanabe Hospital, Osaka, Japan.

[4]Department of Nephrology and Hypertension, Kawasaki Medical School, Kurashiki, Japan.

[5]Department of Cardiovascular Therapeutics, Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan.

* Corresponding Author

E-mail: ichibun@cc.okayama-u.ac.jp

Abstract

Background: The *klotho* gene was identified as an "aging-suppressor" gene that accelerates arterial calcification when disrupted. Serum and vascular klotho levels are reduced in patients with chronic kidney disease, and the reduced levels are associated with arterial calcification. Intake of eicosapentaenoic acid (EPA), an n-3 fatty acid, reduces the risk of fatal coronary artery disease. However, the effects of EPA on arterial calcification have not been fully elucidated. The aim of this study was to determine the effect of EPA on arterial calcification in *klotho* mutant mice.

Methods and Results: Four-week-old *klotho* mutant mice and wild-type (WT) mice were given a diet containing 5% EPA (EPA food, *klotho* and WT: n=12, each) or not containing EPA (control food, *klotho* and WT: n=12, each) for 4 weeks. Calcium volume scores of thoracic and abdominal aortas assessed by computed tomography were significantly elevated in *klotho* mice after 4 weeks of control food, but they were not elevated in *klotho* mice after EPA food or in WT mice. Serum levels of EPA and resolvin E1, an active metabolite of EPA, in EPA food-fed mice were significantly increased compared to those in control food-fed mice. An oxidative stress PCR array followed by quantitative PCR reveled that NADPH oxidase-4 (*NOX4*), an enzyme that generates superoxide, gene expression was up-regulated in arterial smooth muscle cells (SMCs) of *klotho* mice. Activity of NOX was also significantly higher in SMCs of *klotho* mice than in those of WT mice. EPA decreased expression levels of the NOX4 gene and NOX activity. GPR120, a receptor of n-3 fatty acids, gene knockdown by siRNA canceled effects of EPA on NOX4 gene expression and NOX activity in arterial SMCs of *klotho* mice.

Conclusions: EPA prevents arterial calcification together with reduction of NOX gene expression and activity via GPR120 in *klotho* mutant mice.

Introduction

Vascular calcification increases with aging and is highly prevalent in patients with atherosclerosis, diabetes mellitus and chronic kidney disease (CKD) [1]. Coronary artery calcium assessed by computed tomography (CT) provides independent incremental information in addition to traditional risk factors for the prediction of coronary heart disease and all-cause mortality [2, 3].

The *klotho* gene was identified as an "aging-suppressor" gene in mice, and it was shown that disruption of the gene results in acceleration of arterial calcification [4]. We and other investigators have reported that expression levels of serum and local vascular klotho are reduced in patients with CKD and that the decrease in expression level of klotho is associated with arterial calcification and stiffness in patients with CKD [5-7].

Intake of eicosapentaenoic acid (EPA), an n-3 fatty acid, reduces the risk of fatal coronary artery disease [8]. Several studies have revealed that EPA prevents vascular calcification. EPA attenuates arterial medial calcification in warfarin-induced rat models.[9] EPA prevents vascular calcification by inhibiting palmitic acid-induced mineralization of human arterial smooth muscle cells (SMCs) [10]. However, the effects of EPA on arterial calcification such as an association with klotho have not been fully elucidated.

The aim of this study was to determine the effect of EPA on arterial calcification assessed by CT in *klotho* mutant (*kl/kl*) mice. Furthermore, since oxidative stress is associated with the development of vascular calcification [11, 12], we assessed the effects of EPA on gene expression related to oxidative stress in SMCs of *kl/kl* mice.

Materials and Methods

Animals

Klotho homozygous mutant (*kl/kl*) mice were purchased from CLEA Japan. Four-week-old klotho mutant (*kl/kl*) mice (n=24, 12 males & 12 females) and wild-type (WT) mice (n=24, 12 males & 12 females) were given a diet containing 5% EPA (Mochida Pharmaceutical Co. Ltd) (EPA food, klotho and WT: n=12, each) or not containing EPA (control food, klotho and WT: n=12, each) for 4 weeks. All animal protocols were approved and conducted according to the recommendations of Okayama University on Animal Care and Use. The animal procedures performed conform to the NIH guidelines (Guide for the Care and Use of Laboratory Animals).

CT Image acquisition and aortic calcification volume quantification

The mice were anesthetized with inhalation of isoflurane. Image acquisitions were performed using multi-detector CT (FX3000 Pre-Clinical Imaging System, TriFoil Imaging Inc.) before and after 4 weeks of feeding. All images were acquired during an inspiratory breath hold, with tube voltage of 120 kV and single-slice thickness of 192 μm.

Calcium volume score of the thoracic and abdominal aorta was calculated by multiplying the number of voxels (Vn) with the voxel volume (Vv)[13] using the volume-rendering method by extracting the area ≥400 Hounsfield units within the entire aorta.

Serum levels of EPA, arachidonic acid, inorganic phosphorus, calcium and resolvin E1

Serum levels of EPA and arachidonic acid (AA) were measured by gas chromatographic assay (SRL Inc. Tokyo). Serum inorganic phosphorus (Pi) levels were measured by method using molybdate (SRL Inc. Tokyo). Serum levels of calcium (Ca) were determined by method using arsenazo III (SRL Inc. Tokyo). Serum mouse resolvin E1 levels were measured using a commercially available enzyme-linked immunosorbent assay kit (MyBioSource Inc., San

Diego, USA).

Culture of arterial SMCs

After CT image acquisitions, all animals were anaesthetized and euthanized with an intraperitoneal injection of pentobarbital (50 mg/kg). Then the thoracic and abdominal aortas were removed from *kl/kl* mice and WT mice. SMCs were isolated from the thoracic and abdominal aortas by the explant culture method as described previously [14-16]. Thoracic and abdominal aortas were disaggregated with collagenase and cut into 2-mm-long sections, and then the adventitia layer was removed. Vessels were plated on a 6-well plate with Dulbecco's modified Eagle's medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Sigma) and 0.1 mg/mL kanamycin (Sigma) and incubated in a humidified 5% CO_2 atmosphere at 37°C. The culture medium was changed every 3 days. After reaching confluence, the cells were subcultured by treatment with trypsin (0.05%)/ethylenediaminetetraacetic acid (EDTA) (0.02%). Cells between passages 3 to 5 were used for all experiments.

Mouse oxidative stress PCR array and quantitative PCR

Oxidative stress-focused gene expression profiling of SMCs of a *kl/kl* mouse and a WT mouse was performed with the RT^2 Profiler PCR Array System using the mouse oxidative stress PCR array (SABiosciences, a QIAGEN company) according to the manufacturer's instructions. The array measures 84 key genes involved in oxidative stress. Total RNA from arterial SMCs was extracted using RNeasy Mini Kit (QIAGEN). Complementary DNA was synthesized from 1 μg of total RNA using ReverTra Ace® (Toyobo Life Science, Tokyo) as prescribed in the manual and subjected to PCR amplification. Expression of mRNA was

measured by reverse transcription PCR (RT-PCR) using an ABI PRISM 7300 sequence detector system (Applied Biosystems).

For quantitative PCR, arterial SMCs were reseeded in a 10-cm culture dish at a density of 5 x 10^4 cells/well. After 24 hours, arterial SMCs were treated with EPA (20 μmol/L) (Sigma) dissolved in DMSO (Sigma) or 0.08% DMSO as a control. After 24 hours of incubation, total RNA was extracted from the SMCs and complementary DNA was synthesized as described above. Quantitative RT-PCR was performed with primers for cytoglobin (*Cygb*) (PPM28233A, SABiosciences, a QIAGEN company), glutathione peroxidase 3 (*GPX3*) (PPM06171A, SABiosciences, a QIAGEN company) or *GAPDH* (PPM02946E, SABiosciences, a QIAGEN company) in combination with RT^2 SYBR® Green qPCR Master Mix (SABiosciences, a QIAGEN company). Expression of mRNA was measured by RT-PCR using an ABI PRISM 7300 sequence detector system (Applied Biosystems). The quantitative PCR data were processed by a standard curve method. Expression levels were normalized against *GAPDH*.

RT-PCR and Quantitative PCR of NAD(P)H Oxidase 4 (NOX4) gene

Arterial SMCs were reseeded in 10-cm culture dish at a density of 5 x 10^4 cells/well. After 24 hours, arterial SMCs were treated with EPA (20 μmol/L) (Sigma) dissolved in DMSO (Sigma) or 0.08% DMSO as a control. After 24 hours of incubation, total RNA was isolated from the SMCs using TRIzol (Life Technologies Japan). Reverse transcriptase reactions were performed using a Ready-To-Go T-Primed First-Strand Kit (GE Healthcare Japan, Tokyo, Japan) for first-strand cDNA synthesis. Real-time quantitative PCR was performed using the ABI Prism 7700 sequence detection system (Life Technologies Japan). Data were expressed as copy number relative to that of 18 S rRNA. The primers and probe used for

TaqMan analysis of mouse Nox4 were described in our previous report [17]. TaqMan probes consist of the fluorophore 6-carboxyfluorescein (FAM) covalently attached to the 5' end of the oligonucleotide probe and the quencher tetramethylrhodamine (TAMRA) at the 3' end. In detail, the primers and probe for mouse NOX4 were as follows: 5'-cctttgcctccattctcaag-3' (forward primer), 5'-caggtctgcaaaccactcaa-3' (reverse primer) and 5'-FAM-ctggctgtgcagggacacgc-TAMRA-3' (TaqMan probe).

Lucigenin chemiluminescence assay of NAD(P)H Oxidase (NOX) activity

Arterial SMCs were prepared in the same manner as that described for quantitative PCR of NOX4. NOX activity of arterial SMCs was measured using lucigenin chemiluminescence (units/min/mg) as described previously [18]. Briefly, proteins from 5×10^4 SMCs were diluted in modified Hepes buffer (140 mmol/L NaCl, 5 mmol/L KCl, 0.8 mmol/L $MgCl_2$, 1.8 mmol/L $CaCl_2$, 1 mmol/L Na_2HPO_4, 25 mmol/L Hepes, and 1% glucose, pH 7.2) and distributed (100 mg per well) onto a 96-well microplate. NADPH (100 μmol/L) and dark-adapted lucigenin (5 μmol/L; Sigma-Aldrich Japan, Tokyo, Japan) were added just before reading. Lucigenin chemiluminescence was recorded for 5 min and was stopped by the addition of 50 mM Tiron (Sigma-Aldrich Japan, Tokyo, Japan) to observe how the chemiluminescence was detected as superoxide. Lucegenin chemiluminescence was expressed as units per minute per milligram of protein (unit/min/mg). The data are shown as relative chemiluminescence intensity to WT Control. Experiments were performed in triplicate.

Expression of G-protein-coupled receptor 120 (GPR120) mRNA

Total RNA was isolated from cultured SMCs using RNeasy Mini Kit (QIAGEN) as

previously described [19]. First-strand cDNA was synthesized using ReverTra Ace® (Toyobo Life Science, Tokyo). The primers for GPR120 were 5'-ccataaatctagtgctcgct-3' (forward primer) and 5'-tgcggaagagtcggtagtct-3' (reverse primer) as previously described [20].

GPR120 knockdown by siRNA

To knock down GPR120, 5 μmol/L small interfering RNA (siRAN, s200889, Ambion) was transfected into mouse vascular smooth muscle cells explanted from the aorta using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instructions. We confirmed Gpr120 downregulation by the PCR method. Total RNA was extracted using Trizol (Invitrogen) and Purelink RNA Mini Kit (Invitrogen). Complementary DNA was synthesized from 1 μg total RNA using Superscript III with Oligo(dT) primers (Invitrogen) according to the manufacturer's instructions and subjected to PCR amplification. Taq DNA polymerase (Roche Applied Science) was used for RT-PCR. PCR products were subjected to electrophoresis in 2% agarose gels and stained with ethidium bromide. Primer pairs were as follows: GPR120 forward, tgcccctctgcatcttgttc; GPR120 reverse, cgcgatgctttcgtgatctg; GAPDH forward, catggccttccgtgttccta; and GAPDH reverse, tgcctgcttcaccaccttct. PCR product sizes were 202 bp and 106 bp, respectively, and the annealing temperature was 60°C.

Statistical analysis

Data are expressed as mean ± standard error (SE). Data that were not normally distributed are expressed as median and interquartile range (IQR: 25%-75%). Statistical analysis was performed by Student's *t* test or the chi-squared test for paired data or one-way ANOVA with comparison of different groups by Dunnett's *post hoc* test. Values of $P < 0.05$ were

considered to be significant.

Results

Changes in calcium volume score

Calcium volume scores were significantly elevated in *kl/kl* mice after 4 weeks of control food (before vs after feeding, $P < 0.05$) (Fig 1B), but they were not elevated in *kl/kl* mice after 4 weeks of EPA food (before vs after feeding, P = NS) (Fig 1D). Calcium volume scores were not changed in WT mice after 4 weeks of control or EPA food (Fig 1A and C). The change in calcium volume score in control food-fed *kl/kl* mice (81 ± 28 mm^3) was significantly greater than that in control food-fed WT mice (8 ± 6 mm^3, $P < 0.005$) and that in EPA-fed *kl/kl* mice (18 ± 17 mm^3, $P < 0.05$) (Fig 1E). Fig 2 and S1 to S4 Movies show representative CT images of the thoracic and abdominal aortas in *kl/kl* and WT mice. Arterial calcification was elevated in klotho mice after 4 weeks of control food, but EPA prevented arterial calcification in *kl/kl* mice.

Serum levels of EPA, AA, P, Ca and resolvin E1

Serum levels of EPA, arachidonic acid (AA), inorganic phosphorus (P) and calcium (Ca) (Table 1). Serum levels of EPA in EPA-fed *kl/kl* and WT mice were significantly increased compared to those in control-fed *kl/kl* and WT mice, and serum levels of AA in EPA-fed *kl/kl* and WT mice were significantly decreased compared to those in control-fed *kl/kl* and WT mice. The ratios of EPA to AA (EPA/AA) in EPA-fed *kl/kl* and WT mice were significantly larger than those in control-fed *kl/kl* and WT mice.

Serum levels of P in control-fed klotho mice were increased compared to those in control-fed WT mice. EPA intake did not change the levels in klotho and WT mice. These

results indicate that preventative effects of EPA on arterial calcification are not due to effects for negative phosphate balance.

Resolvin E1 is a lipid-derived mediator that is endogenously synthesized from EPA and is generated in response to inflammation and enhances the resolution phase of inflammation [21]. We measured serum levels of resolvin E1 in control food-fed and EPA food-fed WT mice. Serum levels of resolvin E1 in EPA-fed mice (n = 5) were significantly increased compared to those in control-fed mice (n = 6) (control: 1555±95 versus EPA: 1874±56 pg/mL, P = 0.01).

Gene Expression and Activity of NOX

To further assess the mechanism of arterial calcification on *kl/kl* mice, we investigated the involvement of oxidative stress in arterial SMCs using the mouse oxidative stress and antioxidant defense PCR array of RT^2 Profiler PCR Array. Expression levels of apolipoprotein E (*Apoe*), *NOX4*, uncoupling protein 2 (*Ucp2*), heat shock protein 1A (*Hspa1a*), flavin-containing monooxygenase 2 (*Fmo2*), cytoglobin (*Cygb*) and glutathione peroxidase 3 (*GPX3*) genes in arterial SMCs of a *kl/kl* mouse were upregulated compared to those in arterial SMCs of a WT mouse (Table 2). To investigate the involvement of the top 2 upregulated genes, we performed quantitative RT-PCR of *Cygb*, a globin molecule with a protective function during oxidative stress, and *GPX3*, an enzyme having anti-oxidative activity, in arterial SMCs treated with EPA and not treated with EPA. Expression levels of the *Cygb* and *GPX3* genes were significantly higher in arterial SMCs of *kl/kl* mice than in those of WT mice (Figs 3A and B). However, EPA did not decrease expression levels of the *Cygb* and *GPX3* genes.

NOX is an enzyme that produces superoxide and plays a pivotal role in generation of

oxidative stress in vascular SMCs [22]. Other upregulated genes in PCR array are related to proteins that have antioxidant properties. To clarify the involvement of NOX, we performed quantitative RT-PCR of *NOX4*, a component of NOX, and measured NOX activity in arterial SMCs treated with EPA and not treated with EPA. Expression level of the *NOX4* gene and activity of NOX were significantly higher in arterial SMCs of *kl/kl* mice than in those of WT mice (Figs 3C and D). EPA decreased expression levels of the *NOX4* gene and NOX activity.

GPR120 and NOX

G-protein-coupled Receptor 120 (GPR120) is a receptor for n-3 fatty acids [20]. Activation of GPR120 by n-3 fatty acids inhibits inflammation cascades and preserves insulin sensitivity. We hypothesized that inhibitory effects of EPA on NOX expression and activity might be actions on arterial SMCs via GPR120. mRNA of the gene encoding GPR120 was expressed in arterial SMCs obtained from *kl/kl* and WT mice (Fig 4A).

Next, we investigated effects of *GPR120* gene knockdown on *NOX4* gene expression and NOX activity in arterial SMCs of *kl/kl* mice. GPR120 downregulation by siRNA canceled the effects of EPA on *NOX4* gene expression and NOX activity in arterial SMCs of *kl/kl* mice (Fig 4B, C and D).

Discussion

Two major findings were obtained in the present study. First, EPA intake prevented arterial calcification in *klotho* mutant mice. Second, *NOX* gene expression and activity were elevated in arterial SMCs of *klotho* mutant mice and EPA reduced them via GPR120.

Elevated oxidative stress is associated with the development of vascular calcification

[11, 12]. Oxidative stress induces vascular SMC calcification by upregulation of Runx2, which is mediated by activation of the AKT/FOXO1/3 signaling axis. Although there are several enzymatic sources of reactive oxygen species (ROS) in vascular cells, NOX is thought to be a major enzymatic source of ROS in atherosclerosis [23]. Gao et al reported that *klotho* deficiency upregulated NADPH oxidase activity and superoxide production in the media of aortas[24]. We also showed that NOX gene expression and activity were elevated in arterial SMCs of *klotho* mutant mice. Although the precise mechanism underlying the inhibitory effects of EPA on vascular calcification remains unclear, reduction of NOX activity by EPA might play an important role in prevention of vascular calcification in *klotho* mutant mice.

Changes of NOX4 gene expression and NOX activity were minor in our study using a cell culture system. Another mechanism might also contribute to the prevention of vascular calcification *in vivo*. Serum levels of resolvin E1, a bioactive metabolite of EPA, were increased in EPA food-fed mice. Resolvin E1 is responsible for facilitating the resolving phase of acute inflammation [25]. Furthermore, resolvin E1 reduces oxidative stress by suppressing NOX activation [26]. These effects might contribute to the prevention of vascular calcification. Further studies are needed to clarify this point.

Coronary artery calcium assessed by multi-detector CT reflects the plaque burden of coronary arteries, and it is an independent predictor of cardiovascular events and is associated with the incidence of congestive heart failure [2, 27]. Furthermore, one of the most appealing features of CT is the potential to detect the progression or regression of coronary atherosclerotic disease noninvasively [28]. CT also enables evaluation of vascular calcification in living mice [29]. We assessed changes in the degree of arterial calcification by multi-detector CT as recent clinical situations in this study. Our study showed that CT is

useful for detecting the changes over time and by treatment in coronary calcification of living mice.

In the Japan EPA lipid intervention study (JELIS) in patients with hypercholesterolemia, on-treatment mean plasma EPA concentrations and EPA/AA were 170 μg/mL and 1.21, respectively in patients treated with EPA (1800 mg/day) [30]. Therefore, serum levels of EPA in EPA-fed mice were very high in this study (EPA-fed WT mice, median (IQR): 376.9 (142.0), mean ± SD: 373.4 ± 75.4 μg/mL; EPA-fed *kl/kl* mice, median (IQR): 385.8 (169.8), mean ± SD: 393.5 ± 150.1 μg/mL), and EPA/AA ratios in EPA-fed mice were also quite high (EPA-fed WT mice, median (IQR): 11.13 (4.42), mean ± SD: 11.57 ± 2.39; EPA-fed *kl/kl* mice, median (IQR): 8.08 (4.53), mean ± SD: 9.15 ± 3.32). A large amount of EPA might be needed for clinical application of this study. Since it was found in this study that EPA reduced NOX activity via GPR120, novel GPR120 agonists might also be useful for prevention of arterial calcification.

A decrease in the expression level of klotho is associated with arterial calcification and stiffness in patients with CKD [5-7]. Thus, preventative effects of EPA on atrial calcification might be expected in patients with CKD.

In conclusion, EPA intake prevents arterial calcification along with reduction of NOX gene expression and activity via GPR120 in *klotho* mutant mice.

Supporting Information

S1 Movie. CT images of thoracic and abdominal aortas in a *klotho* mutant *(kl/kl)* mouse before control food.

S2 Movie. CT images of thoracic and abdominal aortas in a *klotho* mutant *(kl/kl)* mouse after 4 weeks of control food.

S3 Movie. CT images of thoracic and abdominal aortas in a *klotho* mutant *(kl/kl)* mouse before EPA food.

S4 Movie. CT images of thoracic and abdominal aortas in a *klotho* mutant *(kl/kl)* mouse after 4 weeks of EPA food.

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Author Contributions

Conceptualization: Kazufumi Nakamura.

Data curation: Kazufumi Nakamura, Daiji Miura, Yukihiro Saito, Kei Yunoki, Yasushi Koyama, Minoru Satoh, Megumi Kondo, Kazuhiro Osawa, Omer F Hatipoglu, Toru Miyoshi, Masahi Yoshida and Hiroshi Morita.

Formal analysis: Kazufumi Nakamura, Yasushi Koyama and Minoru Satoh.

Funding acquisition: Kazufumi Nakamura and Hiroshi Ito.

Investigation: Kazufumi Nakamura.

Methodology: Kazufumi Nakamura, Kazuhiro Osawa and Hiroshi Morita.

Project administration: Kazufumi Nakamura and Hiroshi Ito.

Resources: Kazufumi Nakamura and Hiroshi Ito.

Software: Yasushi Koyama.

Visualization: Toru Miyoshi.

Writing-original draft: Kazufumi Nakamura.

Writing–review & editing: Kazufumi Nakamura.

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Disclosures

The investigators have no conflict of interest to declare.

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Figure legends

Fig 1. Changes in calcium volume score. A and B, Calcium volume scores in wild-type (WT) mice (A) and *klotho* mutant (*kl/kl)* mice (B) before and after 4 weeks of control food (n=12, each). C and D, Calcium volume scores in WT mice (C) and *kl/kl* mice (D) before and after 4 weeks of EPA food (n=12, each). E, Changes in calcium volume score in WT mice and *kl/kl* mice before and after 4 weeks of control and EPA food (n=12, each).

Fig 2. Representative CT images of thoracic and abdominal aortas in *klotho* mutant (*kl/kl*) and wild-type (WT) mice. A and B, a WT mouse (A) and a *kl/kl* mouse (B) before and after 4 weeks of control food (S1 and S2 Movies). C and D, a WT mouse (C) and a *kl/kl* mouse (D) before and after 4 weeks of EPA food (Movies S3 and S4).

Fig 3. Gene expression of cytoglobin (*Cygb*), glutathione peroxidase 3 (*GPX3*) and NADPH oxidase (*NOX*) and activity of NOX. A to C, Expression levels of *Cygb* (A), *GPX3* (B) and *NOX4* (C) genes in arterial smooth muscle cells (SMCs) of wild-type (WT) and *klotho* mutant (*kl/kl)* mice treated with EPA and not treated with EPA (control) (n=6, each). D, NOX activity in arterial SMCs of WT and *kl/kl* mice treated with EPA and not treated with EPA (control) (n=6, each).

Fig 4. GPR120 and NADPH oxidase (NOX) in arterial smooth muscle cells. A, Expression of the GPR120 gene. Lane 1, maker; lane 2, wild-type (WT) mouse; lane 3, *klotho* mutant (*kl/kl)* mouse and lane 4, C57/BL6 mouse (positive control). B, GPR120

downregulation by siRNA. Lane 1, si-GPR120 RNA and lane 2, s-scramble RNA. C, Effects of GPR120 gene knockdown on *NOX4* gene expression and NOX activity in arterial SMCs of *kl/kl* mice (n=6, each).

Table 1. Serum levels of EPA, AA, P and Ca

	WT mice			*kl/kl* mice		
	Control food	EPA food	P value	Control food	EPA food	P value
EPA (μg/mL)	7.5 (18.1)	376.9 (142.0)	< 0.001	8.8 (7.9)	385.8 (169.8)	< 0.001
AA (μg/mL)	464.3 (224.5)	32.4 (11.53)	< 0.05	387.0 (191.2)	41.8 (20.0)	< 0.001
EPA/AA	0.01 (0.05)	11.13 (4.42)	< 0.01	0.02 (0.03)	8.08 (4.53)	< 0.001
P (mg/dL)	8.3±0.9	7.9±1.4	NS	16.4±1.5*	15.2±1.0#	NS
Ca (mg/dL)	8.9±0.2	8.9±0.2	NS	9.7±0.3	9.3±0.4	NS

WT: wild-type, *kl/kl*: *klotho* homozygous mutant, EPA: eicosapentaenoic acid, AA: arachidonic acid, P: phosphorus, Ca: calcium, NS: not significant. *$P<0.001$: control food-fed WT vs *kl/kl* mice. #$P<0.001$: EPA-fed WT vs *kl/kl* mice. Data are expressed as median (IQR) or mean ± SE.

Table 2. Genes on RT2 Prodiler PCR-array for which expression is up- or down-regulated in arterial SMCs of *kl/kl* mice

Description	Symbol	Fold Regulation
Albumin	Alb	-3.398
Amyotrophic lateral sclerosis 2 (juvenile) homolog (human)	Als2	-1.007
Aldehyde oxidase 1	Aox1	-2.090
Adenomatosis polyposis coli	Apc	-1.048
Apolipoprotein E	Apoe	2.061
Ataxia telangiectasia and rad3 related	Atr	1.018
Catalase	Cat	-1.376
Chemokine (C-C motif) ligand 5	Ccl5	1.018
Copper chaperone for superoxide dismutase	Ccs	-1.181
Cathepsin B	Ctsb	1.121
Cytochrome b-245, alpha polypeptide	Cyba	-1.403
Cytoglobin	Cygb	16.246
Dynamin 2	Dnm2	1.282
Dual oxidase 1	Duox1	1.959
EH-domain containing 2	Ehd2	-1.089
Eosinophil peroxidase	Epx	-1.053
Excision repair cross-complementing rodent repair deficiency, complementation 2	Ercc2	1.045
Excision repair cross-complementing rodent repair deficiency, complementation 6	Ercc6	-1.154
Fanconi anemia, complementation group C	Fancc	-1.069
Flavin containing monooxygenase 2	Fmo2	3.828
Ferritin heavy chain 1	Fth1	1.034
Glutamate-cysteine ligase, catalytic subunit	Gclc	-2.853
Glutamate-cysteine ligase, modifier subunit	Gclm	1.125
Glutathione peroxidase 1	Gpx1	1.342
Glutathione peroxidase 2	Gpx2	-1.979
Glutathione peroxidase 3	Gpx3	16.279
Glutathione peroxidase 4	Gpx4	1.317
Glutathione peroxidase 5	Gpx5	-1.777
Glutathione peroxidase 6	Gpx6	-1.204
Glutathione peroxidase 7	Gpx7	1.989
Glutathione reductase	Gsr	1.039
Glutathione synthetase	Gss	1.006
Glutathione S-transferase kappa 1	Gstk1	-1.072
Glutathione S-transferase, pi 1	Gstp1	-1.066
Heme oxygenase (decycling) 1	Hmox1	-1.032
Heat shock protein 1A	Hspa1a	2.692
Isocitrate dehydrogenase 1 (NADP+), soluble	Idh1	1.600
Intraflagellar transport 172 homolog (Chlamydomonas)	Ift172	1.390
Interleukin 19	Il19	-1.147
Interleukin 22	Il22	-1.543
Keratin 1	Krt1	-1.058

Lactoperoxidase	Lpo	-1.640
Myoglobin	Mb	-3.055
Myeloperoxidase	Mpo	1.011
Neutrophil cytosolic factor 1	Ncf1	-2.478
Neutrophil cytosolic factor 2	Ncf2	-1.985
Neuroglobin	Ngb	-1.391
Nitric oxide synthase 2, inducible	Nos2	1.016
NADPH oxidase 1	Nox1	1.826
NADPH oxidase 4	Nox4	2.080
NADPH oxidase activator 1	Noxa1	1.127
NADPH oxidase organizer 1	Noxo1	-1.116
NAD(P)H dehydrogenase, quinone 1	Nqo1	1.624
Parkinson disease (autosomal recessive, early onset) 7	Park7	1.156
Peroxiredoxin 1	Prdx1	-1.205
Peroxiredoxin 2	Prdx2	-1.114
Peroxiredoxin 3	Prdx3	-1.035
Peroxiredoxin 4	Prdx4	1.067
Peroxiredoxin 5	Prdx5	-1.368
Peroxiredoxin 6	Prdx6	-1.494
Prion protein	Prnp	1.454
Proteasome (prosome, macropain) subunit, beta type 5	Psmb5	-1.160
Prostaglandin-endoperoxide synthase 1	Ptgs1	-1.029
Prostaglandin-endoperoxide synthase 2	Ptgs2	-2.986
Recombination activating gene 2	Rag2	-11.046
RecQ protein-like 4	Recql4	1.091
Stearoyl-Coenzyme A desaturase 1	Scd1	1.491
Serine (or cysteine) peptidase inhibitor, clade B, member 1b	Serpinb1b	1.938
Solute carrier family 38, member 1	Slc38a1	1.162
Superoxide dismutase 1, soluble	Sod1	-1.258
Superoxide dismutase 2, mitochondrial	Sod2	1.293
Superoxide dismutase 3, extracellular	Sod3	1.470
Sequestosome 1	Sqstm1	-1.400
Sulfiredoxin 1 homolog (S. cerevisiae)	Srxn1	-1.423
Thyroid peroxidase	Tpo	-1.223
Thioredoxin 1	Txn1	-1.687
Thioredoxin interacting protein	Txnip	-1.203
Thioredoxin reductase 1	Txnrd1	-1.399
Thioredoxin reductase 2	Txnrd2	-1.518
Thioredoxin reductase 3	Txnrd3	1.316
Uncoupling protein 2 (mitochondrial, proton carrier)	Ucp2	2.547
Uncoupling protein 3 (mitochondrial, proton carrier)	Ucp3	-1.173
Vimentin	Vim	1.370
Xeroderma pigmentosum, complementation group A	Xpa	1.372

Control food

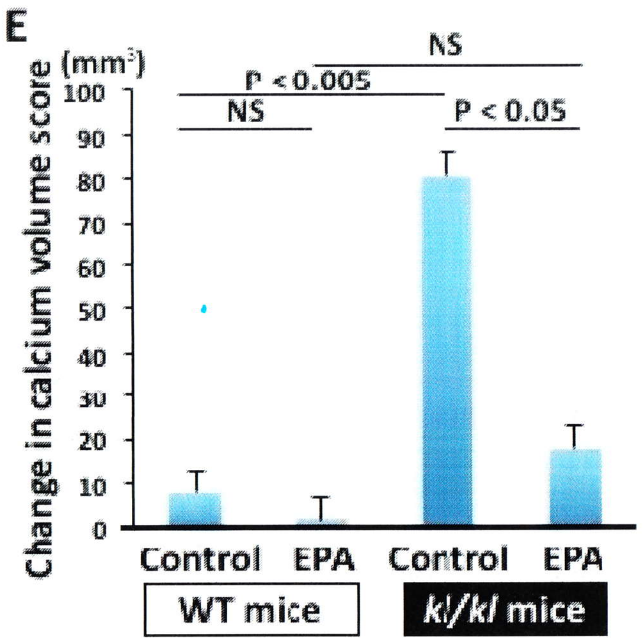
A (mm³) WT mice
Calcium volume score
500 400 300 200 100 0
NS
before after
number 12 12
mean ± SE 1 ± 1 9 ± 6

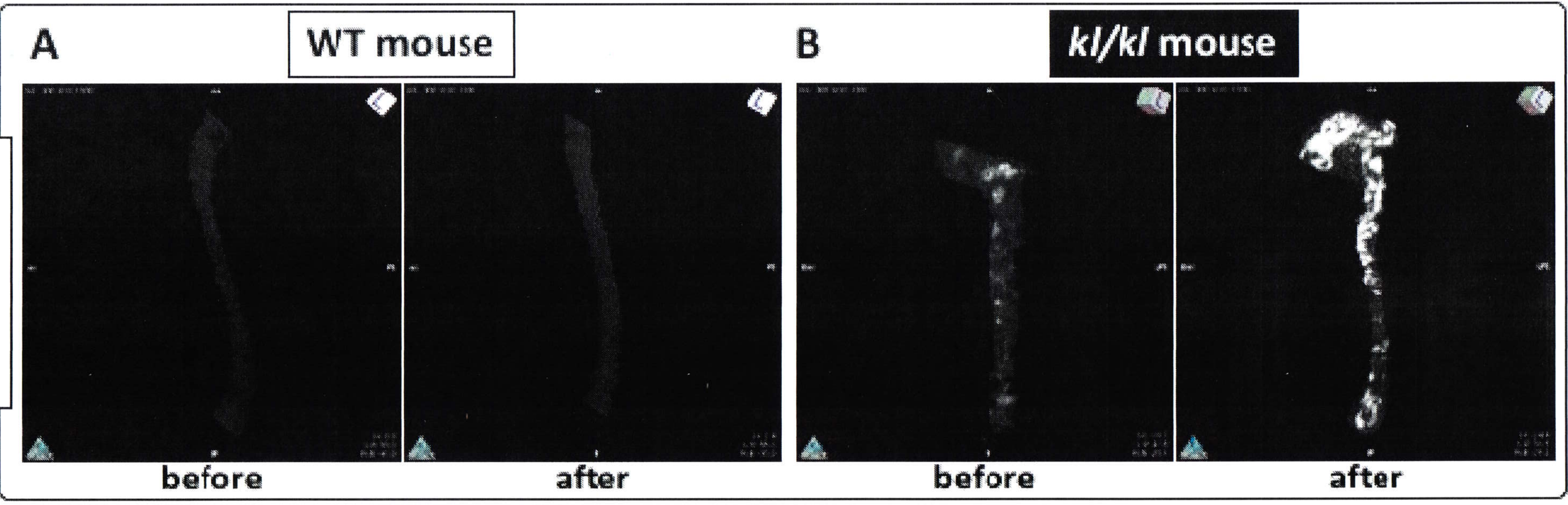
B (mm³) *kl/kl* mice
Calcium volume score
500 400 300 200 100 0
P < 0.05
before after
12 12
43 ± 12 124 ± 24

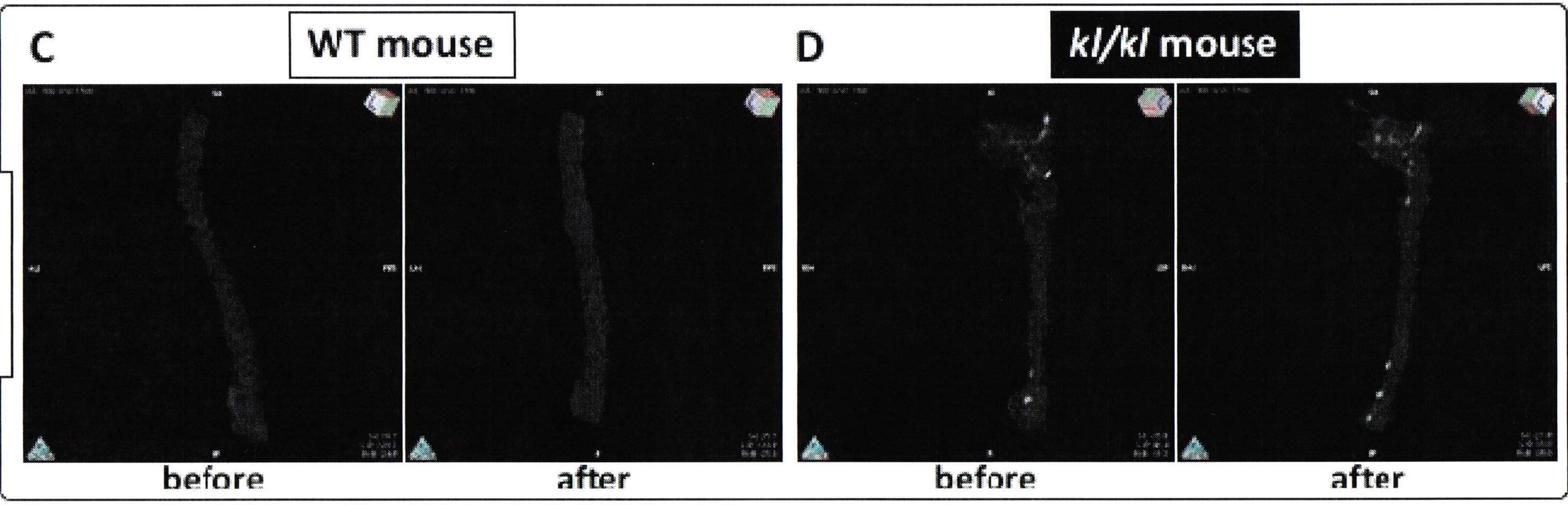
EPA food

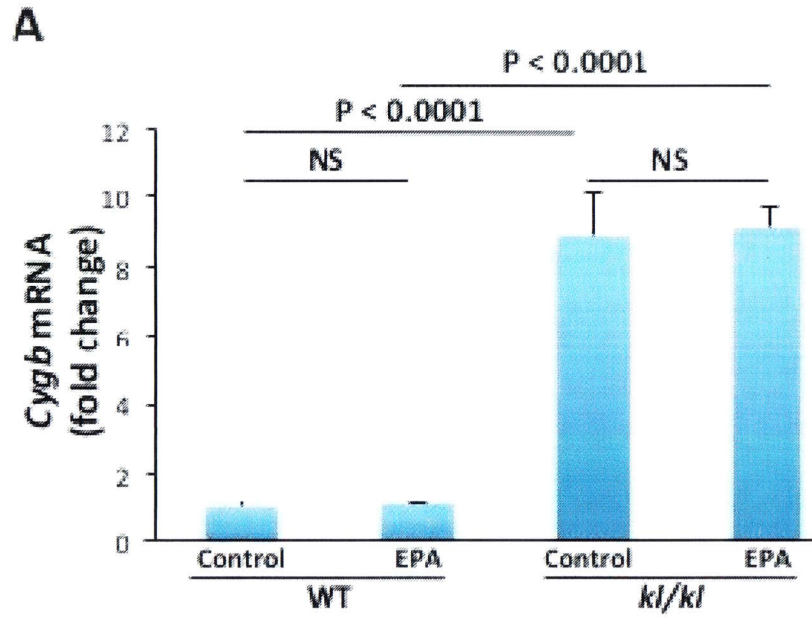
C (mm³) WT mice
Calcium volume score
500 400 300 200 100 0
NS
before after
number 12 12
mean ± SE 10 ± 9 12 ± 5

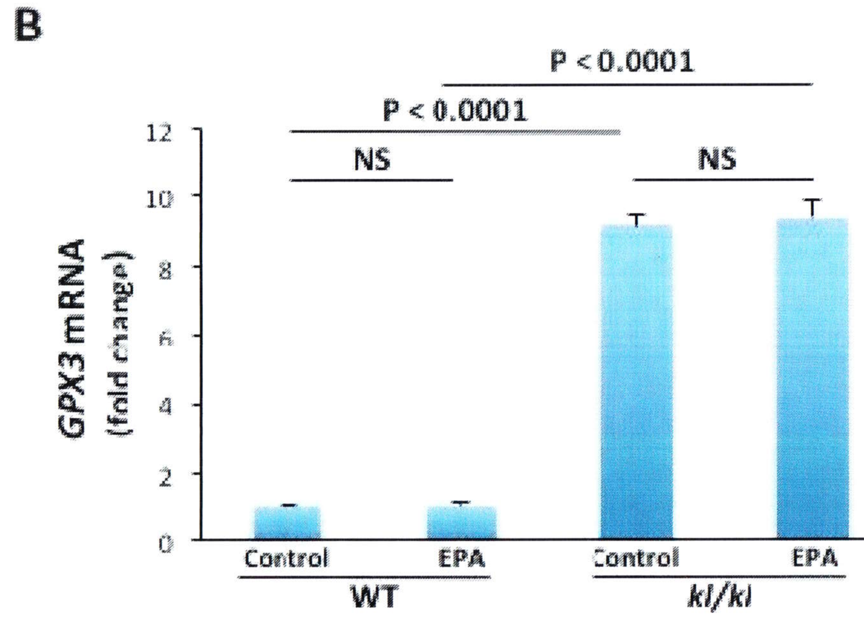
D (mm³) *kl/kl* mice
Calcium volume score
500 400 300 200 100 0
NS
before after
12 12
75 ± 26 93 ± 23

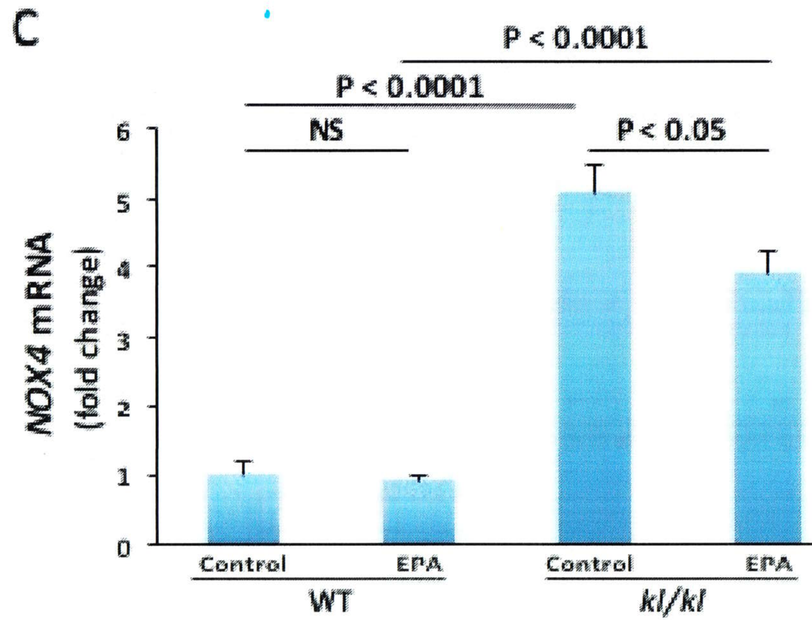


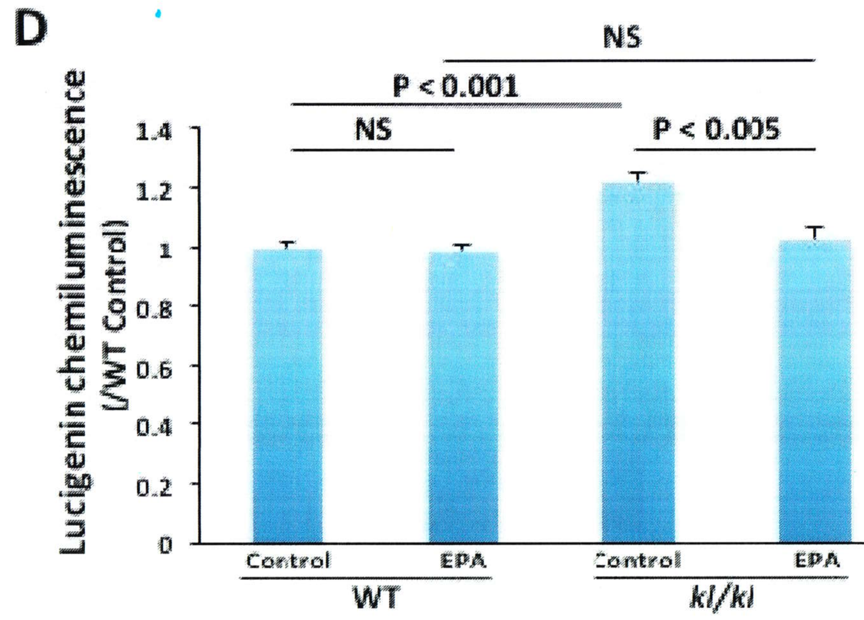

Control food
A
WT mouse
before
after
B
kl/kl mouse
before
after


EPA food
C
WT mouse
before
after
D
kl/kl mouse
before
after

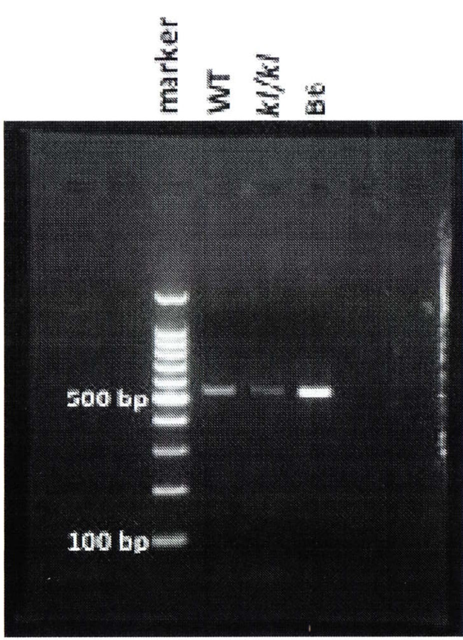

A
P < 0.0001
P < 0.0001
NS
NS
Cygb mRNA
(fold change)
0
2
4
6
8
10
12
Control
EPA
Control
EPA
WT
kl/kl


B
P < 0.0001
P < 0.0001
NS
NS
GPX3 mRNA
0
2
4
6
8
10
12
Control
EPA
EPA
WT
kl/kl


C
P < 0.0001
P < 0.0001
NS
P < 0.05
NOX4 mRNA
(fold change)
0
1
2
3
4
5
6
Control
EPA
Control
EPA
WT
kl/kl


D
NS
P < 0.001
NS
P < 0.005
Lucigenin chemiluminescence
(/WT Control)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
Control
EPA
EPA
WT
kl/kl

A



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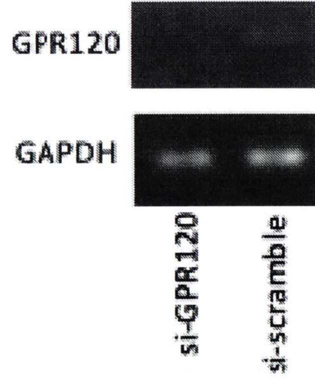

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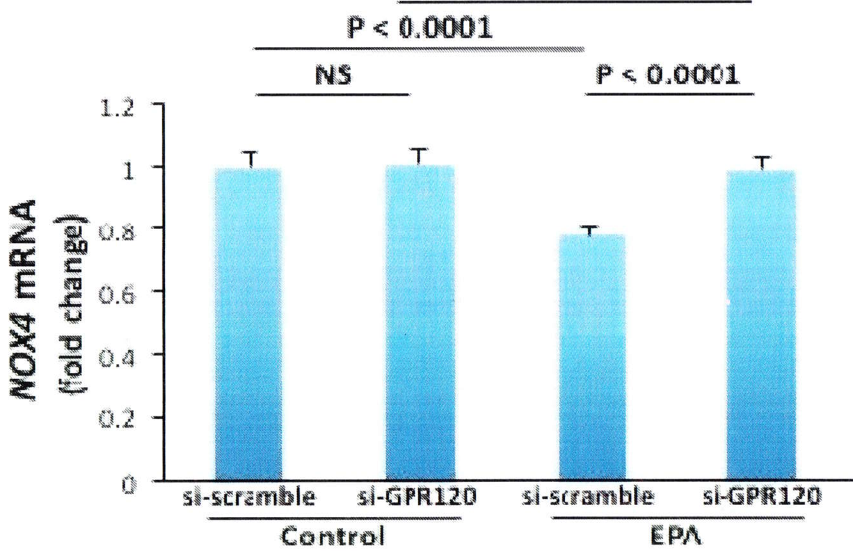

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