様　式　Ｃ－１９、Ｆ－１９－１、Ｚ－１９　（共通）

科学研究費助成事業　　研究成果報告書

令和　５ 年　 ６ 月 ２２ 日現在

機関番号：８４４０４
研究種目：基盤研究(B)（一般）
研究期間：2019～2022
課題番号：１９Ｈ０３４０５

研究課題名（和文）Influence of non-vascular cells in accelerated coronary aging in diabetes

研究課題名（英文）Influence of non-vascular cells in accelerated coronary aging in diabetes

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交付決定額（研究期間全体）：（直接経費）　　13,100,000円

研究成果の概要（和文）：糖尿病は、過剰な酸化物質が抗酸化力を上回り、血管の機能障害や血管の希薄化が起こると、血管の老化を加速させる。我々は、シンクロトロン放射光イメージング法やRNAseqやプロテオーム解析を用いてインスリン抵抗性が、Nox2やiNOSの活性化を介した酸化物質の上昇、代謝の柔軟性の低下、ミトコンドリア機能障害による冠動脈機能障害を引き起こすことを、老化のマウスモデルで実証した。

研究成果の学術的意義や社会的意義
我々の研究は、マウスにおいて優れたイメージング技術により、カロリー過多や塩分多量摂取などの食生活の乱れによる生活習慣病が、心臓の微小血管の早期老化を引き起こし、運動やストレス時に心臓の機能を低下させることを明らかにした。また、運動がこの血管機能障害を改善することを示し、治療のターゲットを特定した。

研究成果の概要（英文）：Diabetes accelerates vascular ageing when excess oxidants exceed antioxidant capacity, vascular dysfunction and vessel rarefaction. We tested whether Nox2 contributes to coronary dysfunction in diet induced early-stage diabetes induced by high fat diet (HFD) and increased salt intake in mice treated with and without apocynin. Synchrotron microangiography revealed that the coronary capacity to produce NO (nitric oxide) was diminished by insulin resistance, suggesting Nox2 overactivation in insulin resistance reduces NO bioavailability. In SAMP8 mice glycolysis was inhibited and abnormal purine metabolism increased xanthine oxidase activation on HFD leading to microvascular dysfunction. Mice developed mild coronary dysfunction due to elevated oxidants, metabolic inflexibility and mitochondrial dysfunction. We conclude that insulin resistance greatly increases endothelin production through Nox2 to reduce NO bioavailability. iNOS upregulation promoted p53 activation in senescent mice.

研究分野：循環器生理学

キーワード：coronary circulation　diabetes　senescence　inflammation　oxidative stress　nitric oxide

１．研究開始当初の背景

A sedentary lifestyle, excess calorie and salt intake induce premature vascular aging and cardiovascular disease through chronic inflammation and oxidative stress by inducing a senescence associated secretory phenotype (SASP). It was established that reactive oxygen species (ROS) induced DNA damage and subsequent p53/NF-κβ activation and thereby cell senescence. Internationally, various groups had demonstrated a role for coronary microvascular dysfunction and nitric oxide (NO) depletion in diastolic dysfunction in established heart failure associated with diabetes, vascular aging and the initiation of insulin resistance (1, 2). However, previous research on cardiac senescence did not consider the relative contributions of non-vascular cell types in the induction of vascular aging before diabetes becomes advanced. Many proposed that coronary endothelial cells (EC) were the major source of oxidative stress causing coronary endothelial dysfunction without ever investigating this assumption. Earlier research indicated that stress activation of p53 in mouse cardiomyocytes (CM) increased ROS production through monoamine oxidase-A (MAO-A) (3). On the other hand, upregulation and overactivation of NADPH oxidase-2 (Nox2) in EC and immune cells in various pathological states was considered a major source of ROS.

２．研究の目的

The main objective was to determine whether coronary vascular aging in diabetes originates from early inflammation and oxidative stress that develops in CM or cells within the extracellular matrix and is then exacerbated by EC senescence and reduced capacities to produce NO and other vasodilators with aging in sedentary rodents. To achieve this aim, we utilized senescence accelerated mouse prone-8 (SAMP8, non-diabetic model) and resistant-1 (SAMR1) mice, a mouse model of diet-induced obesity and insulin resistance and both prediabetic and diabetic Goto-Kakizaki (GK) rats.

３．研究の方法

Mice and rats were maintained on special diets to simulate the spectrum of lifestyle disease from insulin resistance to type-2 diabetes and cardiac and coronary function were assessed over the specified time courses indicated below with echocardiography and synchrotron based microangiography. Whole left ventricles or cell separated fractions were used for molecular investigations. In this research we investigated the origins of oxidative stress and inflammation with cell separation technology, metabolomic and proteomic analyses, qPCR investigation of gene expression changes, fluorescence tracer imaging approaches to dissect the crosstalk mechanisms between the coronary circulation and myocardium during accelerated aging in rodent models. The main rodent models utilized were as follows;

(1) SAMP8 and SAMR1 male mice on a high fat diet (HFD) with 1% NaCl drinking water for 24 weeks, including SAMP8 mice chronically treated with MAO-A inhibitor (clorgyline) or Nox2 inhibitor (apocynin) to inhibit CM and EC/immune cell sources of ROS respectively.

(2) B6D2 hybrid male mice were for 8-16 weeks on either HFD or HFD in combination with high sugar diet (HFHSD) to induce insulin resistance. On the basis of findings in (1) we included a group of apocynin-treated mice on HFD.

(3) Male Wistar and GK rats on a high salt (6% NaCl) diet.

４．研究成果

We found that the type of lifestyle disease diet influenced vascular and cardiac function outcomes differentially in our models dependent on the presence of insulin resistance and whether it progressed to type-2 diabetes. Our main findings suggest that diet associated metabolic dysregulation in CMs contribute to the reduction of NO bioavailability for coronary vasodilation and at the same time increase vasoconstrictor production even in the absence of insulin resistance. While there was no role for Nox2 overactivation in coronary microvascular dysfunction due to senescence this role changed and Nox2 greatly contributed to microvascular dysfunction in the insulin resistant state. The main findings are briefly summarized below.

(1) In the absence of insulin resistance, HFD was found to diminish coronary arteriole-small artery NO-mediated vasodilation in SAMP8 mice relative to SAMR1 mice (Figure 1). While there were no indications of heart failure in SAMP8 vehicle treated mice and basal coronary perfusion was well maintained, selective blockade of coronary vasodilators (EDH and prostanoids) revealed a reduced ability to produce NO in microvessels and endothelium dependent vasoconstrictor production. Notably, MAO-A inhibition did not alter the microvascular dysfunction, while Nox2 inhibition evoked pronounced vasoconstriction in the absence of EDH and prostanoids (data not shown). The latter suggests that Nox2 is not appreciably upregulated in the heart in this SAMP8 model and confirms that Nox2 is an important cofactor for eNOS coupling to vasodilation. High intensity interval training of SAMP8 HFD mice largely ameliorated coronary

microvascular dysfunction in the absence of insulin resistance (Figure 2). We then established the molecular basis of these signaling pathway changes using immunoblotting and metabolome analyses.

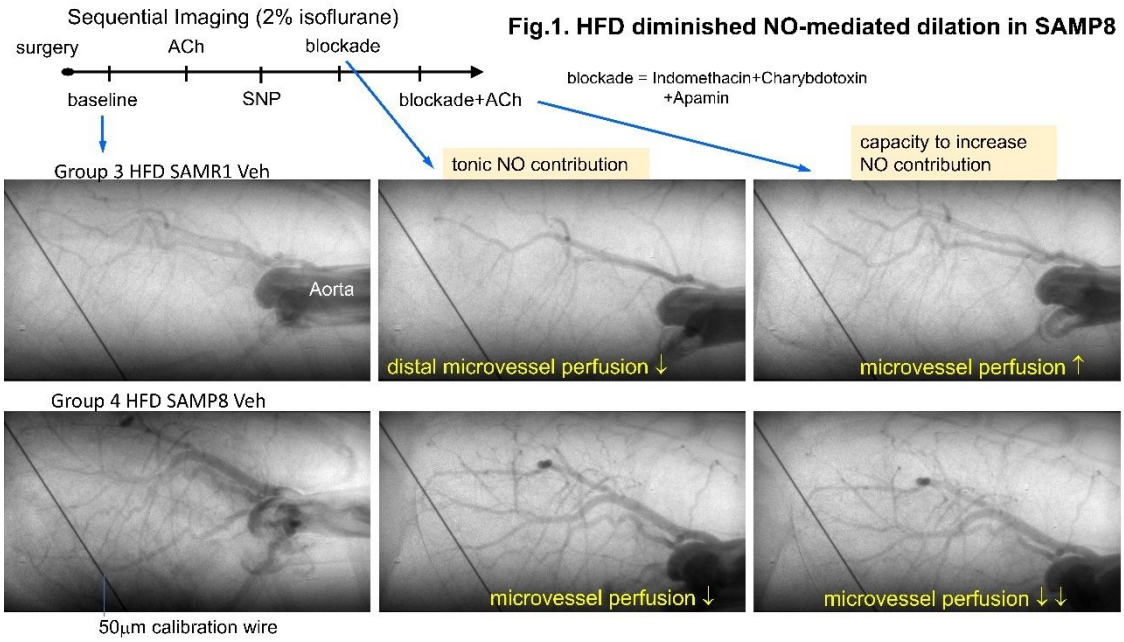


Fig.1. HFD diminished NO-mediated dilation in SAMP8

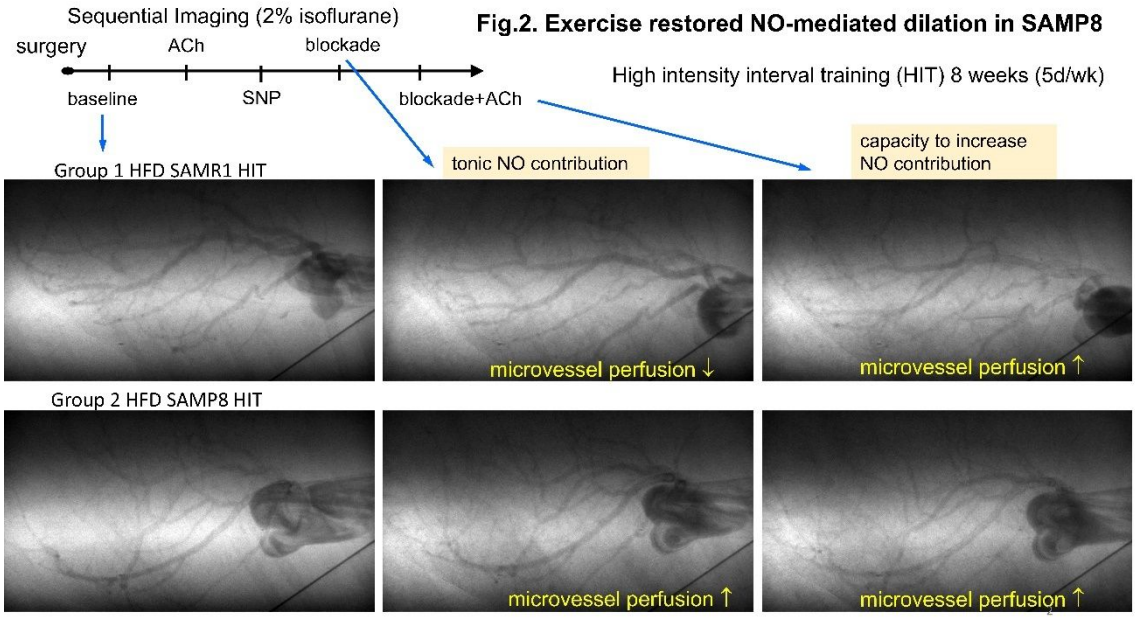


Fig.2. Exercise restored NO-mediated dilation in SAMP8

In SAMP mice, in addition to the reported elevation in p53 expression we found that the HFD increased vasoconstrictor factor (ET-1, ROCK2) expression. Acetylation and phosphorylation modifications of p53 protein showed opposite changes in response to HFD. On the other hand, SAMR1 mice showed a compensatory increase in Sirt1 expression that was not observed in SAMP8 mice on the same diet. Metabolome analyses of myocardium showed changes in xanthine oxidase (XO) signaling supporting a role for increased CM activation of XO, but no change in arginase signaling within the vasculature (Figure 3). Immunoblotting of phospho-eNOS revealed that vasodilatory NO signaling was suppressed by HFD, but eNOS was not uncoupled by this diet treatment, and rather L-Arginine and NO bioavailability was reduced in part by XO activation. Nitrosative stress due to iNOS upregulation and 3-nitrotyrosine production due to the HFD was minor, in contrast to a previous study on female SAMP8 mice (4).

(2) HFD treatment in B2D2 hybrid mice increased fasted blood glucose and glucose intolerance consistent with insulin resistance and prediabetes. While B6D2 mice on a normal diet maintained the ability to increase NO-mediated vasodilation in coronary microvessels (not shown) during blockade of other vasodilators this was lost in HFD treated mice (Figure 4, left panels). In contrast to the SAMP8 study, apocynin treated B6D2 mice on HFD maintained NO-mediated vasodilation (Figure 4, right panels). This finding suggests that with insulin resistance development Nox2 activity is greatly potentiated and oxidative stress from vascular origin is exacerbated. RNAseq analysis of myocardium showed that metabolic dysregulation of CMs also likely contributed to the observed ED dysfunction (to be confirmed).

Fig.3. Factors determining Nitric Oxide Bioavailability for Vasodilation: non-diabetic SAMP8 mice

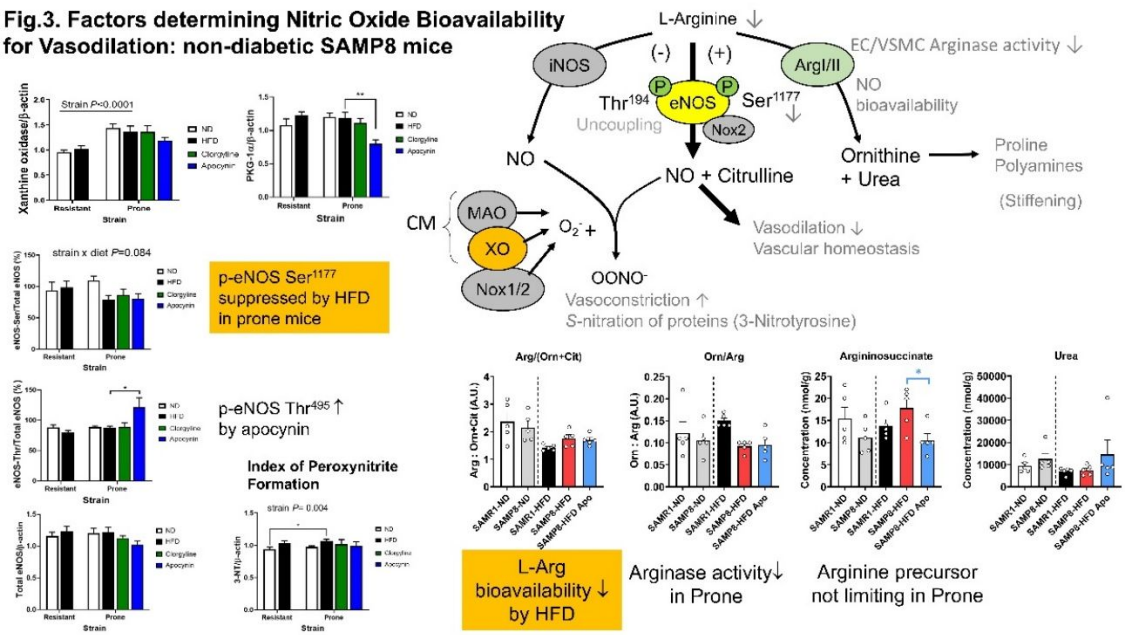
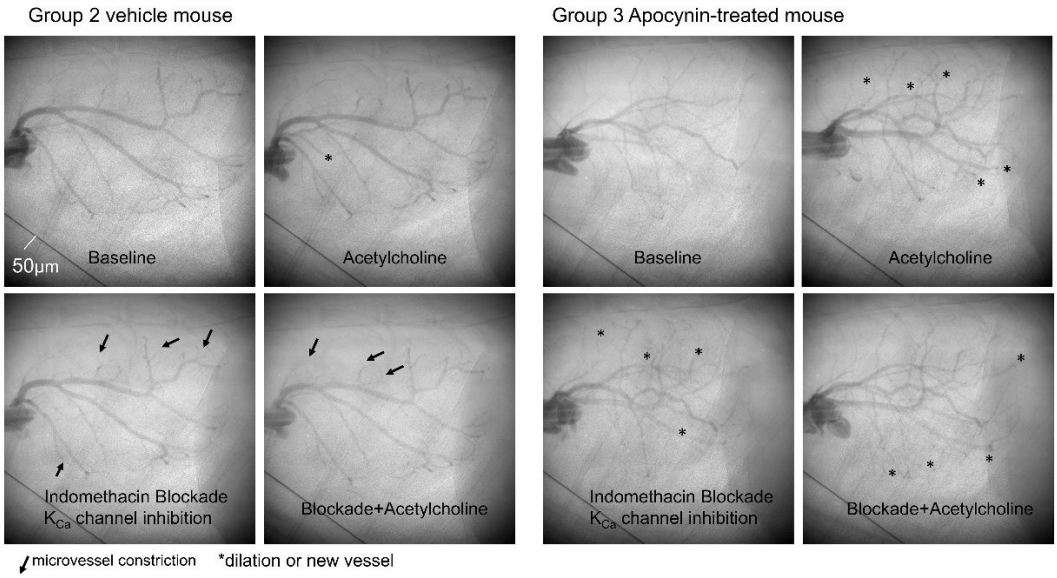
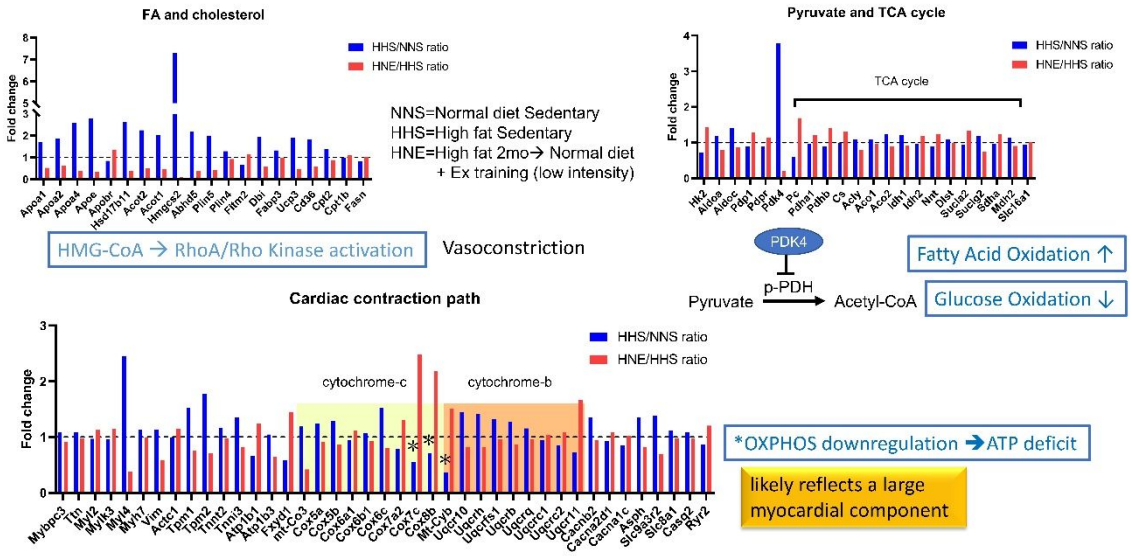


Fig.4. Nox2 Upregulation Reduces Nitric Oxide Bioavailability in insulin resistant B6D2 mice



In follow up experiments in B6D2 mice we found that dietary intervention in combination with low intensity exercise training (treadmill running) after 8wk of HFD evoked distinct changes in protein expression relative to mice that remained sedentary on HFD (Figure 5). Indeed, the differential protein changes in HFD mice relative to normal diet mice indicated that metabolic dysregulation and mitochondrial dysfunction in CMs were pronounced, but also indicated that elevated HMG-CoA levels due to dyslipidemia potentially drives the increase in vasoconstriction through overactivation of RhoA/Rho Kinase signaling (Figure 5, blue bars). In addition, there was a pronounced downregulation of histone H1.2, which suggests that the DNA damage response was impaired by HFD exposure. Notably, these changes in CM proteome were all reversed by the diet intervention with exercise training (Figure 5, red bars). From transcriptome analyses by RNAseq, 49 differentially expressed genes were found to be reversed by diet intervention and exercise training and gene ontology analysis suggests that most of the significant pathway changes were associated with vasculature development and regulation.

Fig.5. Effects of Diet Intervention and Exercise in Diet-induced Obese Mice



(4) High salt diet exacerbated insulin resistance in the prediabetic GK rats, accelerating diabetes progression. Here we showed that sympatho-adrenal overactivation by the high salt consumption increased the proinflammatory environment and ROS generation through PKC and iNOS upregulation and activation, as well as promoting endothelin-1 and Rho Kinase activation and downregulation of eNOS (JT Pearson et al Clinical Science 135, 2021, 327-346). We considered that the proinflammatory environment of the myocardium associated with chronic insulin resistance predisposes the GK rat to exaggerated constrictor tone in the coronary microcirculation, which may be in part due to metabolic dysregulation of non-vascular cells.
(5) Within budget constraints cell separation and isolated CM from young and aged GK and Wistar rats were initiated after repeated delays due to the COVID-2019 pandemic. EC and CM incubation protocols were refined and conditioned media from cells incubated in low and high glucose conditions were collected. However, analysis of cytokine production from isolated CMs remains ongoing to be considered in future research.

The findings described herein for the non-vascular cell contributions to coronary vascular aging in senescent mice and various models of insulin resistance are currently being prepared for multiple manuscript submissions.

〈引用文献〉
(1) T Minamino et al. A crucial role for adipose tissue p53 in the regulation of insulin resistance. Nature Medicine 15(9), 2009, 1082-1087.
(2) G Katsuumi, I Shimizu, Y Yoshida, T Minamino. Vascular senescence in cardiovascular and metabolic diseases. Front. Cardiovasc. Med. 5, 2018, 18.
(3) C Villeneuve et al. p53-PGC-1α pathway mediate oxidative mitochondrial damage and cardiomyocyte necrosis induced by monoamine oxidase-A upregulation: role in chronic left ventricular dysfunction in mice. Antioxidants & Redox Signaling 18(1), 2013, 5-18.
(4) AB Gevaert et al. Endothelial senescence contributes to heart failure with preserved ejection fraction in an aging mouse model. Circ. Heart Failure 10, 2017, e003806.

５．主な発表論文等

〔雑誌論文〕　計3件（うち査読付論文　3件／うち国際共著　3件／うちオープンアクセス　2件）

１．著者名 Mark T Waddingham, Hirotsugu Tsuchimochi, Takashi Sonobe, Ryotaro Asano, Huiling Jin, Connie PC Ow, Daryl O Schwenke, Rajesh Katare, Kohki Aoyama, Keiji Umetani, Masato Hoshino, Kentaro Uesugi, Mikiyasu Shirai, Takeshi Ogo, James T Pearson	４．巻 12
２．論文標題 Using synchrotron radiation imaging techniques to elucidate the actions of hexarelin in the heart of small animal models	５．発行年 2021年
３．雑誌名 Frontiers in Physiology	６．最初と最後の頁 766818
掲載論文のDOI（デジタルオブジェクト識別子） 10.3389/fphys.2021.766818	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

１．著者名 Pearson James T. et al	４．巻 135
２．論文標題 β-blockade prevents coronary macro- and microvascular dysfunction induced by a high salt diet and insulin resistance in the Goto?Kakizaki rat	５．発行年 2021年
３．雑誌名 Clinical Science	６．最初と最後の頁 327～346
掲載論文のDOI（デジタルオブジェクト識別子） 10.1042/CS20201441	査読の有無 有
オープンアクセス オープンアクセスではない、又はオープンアクセスが困難	国際共著 該当する

１．著者名 James T Pearson	４．巻 1
２．論文標題 Ghrelin and vascular protection	５．発行年 2019年
３．雑誌名 Vascular Biology	６．最初と最後の頁 H97-H102
掲載論文のDOI（デジタルオブジェクト識別子） 10.1530/VB-19-0024	査読の有無 有
オープンアクセス オープンアクセスとしている（また、その予定である）	国際共著 該当する

〔学会発表〕　計3件（うち招待講演　0件／うち国際学会　0件）

１．発表者名 James Pearson
２．発表標題 Exercise reverses the sarcomeric and cytoskeletal contributions to diastolic dysfunction in mice with diet-induced insulin resistance and obesity
３．学会等名 第９９回日本生理学会大会
４．発表年 2022年

１．発表者名 James Pearson
２．発表標題 Coronary nitric oxide bioavailability is reduced by xanthine oxidase and nitrosative stress on a high fat diet but restored by high intensity exercise in senescence-accelerated mice
３．学会等名 第９８回日本生理学会大会
４．発表年 2021年

１．発表者名 James Pearson
２．発表標題 Early loss of coronary regulation by endothelium-derived hyperpolarisation factors in diabetes
３．学会等名 第97回日本生理学会大会
４．発表年 2020年

〔図書〕　計0件

〔産業財産権〕

〔その他〕

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６．研究組織

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７．科研費を使用して開催した国際研究集会

〔国際研究集会〕　計0件

８．本研究に関連して実施した国際共同研究の実施状況

共同研究相手国	相手方研究機関