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Original article

# Excessive intake of trans fatty acid accelerates atherosclerosis through promoting inflammation and oxidative stress in a mouse model of hyperlipidemia

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ABSTRACT

*Background:* Epidemiological studies have demonstrated that trans fatty acids (TFAs) are a risk for coronary artery disease. However, the precise mechanism underlying the proatherogenic effect of TFA has not been completely elucidated. To obtain better understanding of the impact of TFA on vascular diseases, this study investigated the effect of TFA on oxidative stress using a mouse model of atherosclerosis.

*Methods:* Low-density lipoprotein (LDL) receptor knockout mice were fed with diet containing 0.5% cholesterol (control), 0.5% cholesterol + 5% elaidic acids (Trans group), and 0.5% cholesterol + 5% oleic acids (Cis group) for 8 weeks. Atherosclerotic lesion and oxidative stress in aortic wall were evaluated. In vitro experiments using smooth muscle cells were performed to corroborate in vivo findings.

*Results:* The atherosclerotic lesion area was significantly larger in Trans group than that in control or Cis group. Lipoprotein fractionation was similar among groups, while plasma oxidized LDL level and superoxide production in the vessel wall were markedly increased in Trans group. Elaidic acids were accumulated in a variety of tissues including liver and adipose tissue, which was associated with the high level of inflammatory cytokines in these tissues and plasma. Aortic wall from Trans group showed augmented expression of reactive oxygen species and NAPDH oxidase (p22phox) in smooth muscle cells. In vitro experiments confirmed that elaidic acids upregulated expression of NADPH oxidase and inflammatory cytokines in cultured smooth muscle cells.

*Conclusion:* Excessive intake of TFA contributes to the progression of atherosclerosis by evoking inflammation and oxidative stress in mice.



## Introduction

The risk of atherosclerotic cardiovascular disease is largely linked to modifiable environmental factors, including diet. Epidemiological investigations from western countries have revealed a significant positive association of cardiovascular disease with the consumption of trans fatty acids (TFAs) [1–3]. Although intake of dietary lipids in Japan is considered to be lower than western countries, we recently reported that serum TFA concentration is elevated in patients with coronary artery disease (CAD) in Japan [4].

TFAs are unsaturated fatty acids with at least one double bond in the trans configuration. It is estimated that TFA contributed up to 4–12% of total dietary fat intake in the US population [5]. The majority of TFA in our diet are industrially produced during the

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partial hydrogenation of vegetable oils, a process that converts unsaturated oils into semisolid fats for use in a variety of foods such as deep-fried fast foods, bakery products, packaged snack foods, margarines, and crackers [6,7].

Among all TFAs, elaidic acid (18:1 trans-9) is known as the main isomer in industrially produced TFAs. In the Seven Countries Study, a strong positive association was observed between 25-year death rates from coronary heart disease and average intake of elaidic acid [8]. Although observational studies indicate that TFA could accelerate atherosclerosis, there are few studies demonstrating the mechanism of dietary TFA on the development of CAD. The purpose of this study was to investigate the impact of dietary TFA on oxidative stress and development of atherosclerosis using a mouse model of hyperlipidemia.

## Materials and methods

### *Materials and animal preparation*

Male low-density lipoprotein receptor-deficient mice (LDLr−/−, Jackson Laboratories, Bar Harbor, ME, USA) on a C57BL/6 genetic background at 6 weeks of age were randomly assigned to one of the following three groups: (1) fed a diet containing 0.5% (w/w) cholesterol (Control group, $n = 10$); (2) fed a diet containing 0.5% (w/w) cholesterol and 5% (w/w) elaidic acid (C18:1, 9-*trans*) (Trans group, $n = 13$); (3) fed a diet containing 0.5% (w/w) cholesterol and 5% (w/w) oleic acid (C18:1, 9-*cis*) (Cis group, $n = 12$). Mice were provided the diet and water ad libitum and maintained on a 12 h light/dark cycle for 8 weeks and euthanized at 14 weeks of age. All animal experiments were conducted according to the guidelines for animal experiments at Kobe University Graduate School of Medicine.

### *Plasma lipids and glucose analysis*

Blood samples were taken by cardiac puncture when mice were euthanized after a 4-h fasting. Plasma levels of cholesterol and triglyceride were measured enzymatically at the Nagahama Life Science Laboratory of Oriental Yeast Co., Ltd. (Shiga, Japan). Blood glucose was measured by OneTouch Ultra glucometer (LifeScan, Wayne, PA, USA). Lipoprotein fractionation analysis was performed by high performance liquid chromatography (HPLC) (LipoSEARCH®) in the Skylight Biotech, Inc. (Akita, Japan) according to the specified procedure [9,10].

### *Blood pressure measurement*

Systolic blood pressure (SBP) was measured using a noninvasive tail-cuff blood pressure machine (BP-98, Softron, Tokyo, Japan). Conscious mice were placed on the warmed platform of the machine, which was maintained at 37 °C, and were allowed to acclimatize to the apparatus for 5 min before the start of measurement. The SBP was taken at least three times per mouse, and then the values were averaged to determine the SBP for the mouse.

### *Histological analysis of atherosclerotic lesions*

Mice were euthanized at the age of 14 weeks and the atherosclerotic lesions were analyzed as described previously [11]. The aortic samples were fixed in 4% paraformaldehyde, embedded in OCT compound (Tissue-Tek, Sakura Finetek, Tokyo, Japan), and sectioned (10-μm thickness). Five consecutive sections (10-μm thickness), spanning 550 μm of the aortic root, were collected from each mouse and stained with Masson trichrome (MT), Elastica van Gieson (EVG), and Oil Red O. For quantitative analysis of atherosclerosis, the total lesion area of five sections from each mouse was measured with Image J (US National Institutes of Health, Bethesda, MD,USA) as reported previously [12].

### *Immunohistochemistry*

Immunohistochemical staining with MOMA-2 (BMA Biomedicals, Augst, Switzerland) of atherosclerotic lesion at the aortic sinus was performed by the labeled streptavidin biotin method as described previously [11]. Quantitative analysis of MOMA-2-immunostaining was shown as a percentage of the positive-stained area in the total atherosclerotic lesion area. Smooth muscle actin (SMA) was stained using anti-SMA-FITC conjugated antibody (Sigma).

### *Quantitative real-time polymerase chain reaction*

Total RNA was extracted from samples of murine aorta, liver, and fat. Quantitative real-time polymerase chain reaction (PCR) was performed as previously reported [13]. Glyceradehyde-3-phosphate dehydrogenase (GAPDH) was used for normalization. The nucleotide sequences of the primers used are shown in Table 1.

### *Detection of superoxide by in situ dihydroethidium method*

Dihydroethidium (DHE, Molecular Probes, Eugene, OR, USA), a superoxide sensitive fluorescent dye, was used to detect superoxide in aorta as previously reported [14]. Briefly, fresh unfixed segment of aorta was made into sections and incubated with 2 μmol/L DHE in a light-protected chamber at 37 °C for 30 min. Sections were visualized with a fluorescence microscope (BZ-8000, KEYENCE, Osaka, Japan), and fluorescence intensities were quantified.

### *Fatty acid analysis with gas chromatography/mass spectrometry*

Nonadecanoic acid (C19:0) was added as an internal standard to each plasma sample (50 μL), followed by total fatty acid extraction, methylester derivatization, and purification using the

**Table 1**
Primers for quantitative real-time PCR.

| Gene | Forward primer (5′-3′) | Reverse primer (5′-3′) |
|---|---|---|
| Mouse GAPDH | TGTGTCCGTCGTGGATCTGA | TTGCTGTTGAAGTCGCAGGAG |
| Mouse IL-1β | TCCAGGATGAGGACATGAGCAC | GAACGTCACACACCAGCAGGTTA |
| Mouse TNFα | ACGGCATGGATCTCAAAGAC | AGATAGCAAATCGGCTGACG |
| Rat GAPDH | AACCCATCACCATCTTCCAGG | GGGGCATCAGCGGAAGG |
| Rat p22phox | CCAATTCCAGTGACAGATGAG | GGGAGCAACACCTTGGAAAC |

GADPH, glyceradehyde-3-phosphate dehydrogenase; IL-1β, INTERLEUKIN-1 beta; TNFα, tumor necrosis factor alpha.

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