Dietary Sodium Nitrite Causes Similar Modifications to Splenic Inflammatory Gene Expression as a High-Fat Diet

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Summary Sodium nitrite (NaNO2) is a widely used food additive. The present study compared the outcomes from intakes of dietary NaNO2 and a high-fat diet (HFD), and assessed their combined effects on inflammatory gene expression in the immune tissues of the mouse. In experiment I, mice were fed a standard low-fat diet (LFD) without or with NaNO2 (0.02 and 0.08%, w/w) for 11 wk. In experiment II, mice were fed an LFD without or with NaNO2 (0.02%) or HFD without or with NaNO2 (0.02%) for 11 wk. Inflammatory gene expression in the immune tissues was then measured. NaNO2 consumption and HFD feeding each resulted in increased splenic mRNAs for cell markers of neutrophils (Ngp, NE, Ly6g, Mpo) and eosinophils (Epo, Ear6), and an S100 family member (S100A8). In contrast, NaNO2 consumption and HFD feeding each resulted in decreased splenic mRNAs for cell markers of macrophages (Emr1, Itgax, CD68, CD206, Dectin-1, TLRs 4, 6, and 7), T- (CD3, CD4), NK- (CD56) and B-cells (CD20, CD40), pro- and anti-inflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ, IL-18, IL-10, TGF-β), interleukin receptor antagonists (IL1ra, IL6ra) and cell adhesion molecules (ICAM-1, VCAM-1). However, dietary NaNO2 combined with HFD feeding caused no further decrease in these transcript levels compared with dietary NaNO2 alone. These NaNO2- or HFD-induced modifications were less profound in the liver and abdominal adipose tissues than in the spleen. These findings indicate that dietary NaNO2 has similar modulatory effects to HFD feeding on splenic inflammatory genes.

Key Words sodium nitrite, high fat diet, spleen, inflammatory gene expression, neutrophils

Sodium nitrite (NaNO2) is a widely used inorganic compound in both the food and drug industries. In the food industry, NaNO2 is used as a color fixing agent and as a preservative in cured meats (processed meats, bacon, hot dogs) and smoked fish (1). In medical applications, NaNO2 is employed as a vasodilator, a bronchodilator and an antidote for cyanide poisoning (2). Both beneficial and harmful effects of NaNO2 consumption have been reported. Dietary NaNO2 supplementation (i.e. 50 mg NaNO2/L in drinking water for 8 wk; 100 mg NaNO2/L in drinking water for 2 mo) is reported to alleviate the metabolic abnormalities associated with type 2 diabetes in rats (e.g., hyperglycemia, insulin resistance and hyperlipidemia) (3, 4). In contrast, long term NaNO2 consumption (20 mg/kg/d, 8 mo) causes histopathologic changes, nitrosative tissue damage, and lipid peroxidation in the livers and kidneys of mice (5).

Although inflammation is important for the innate and adaptive immune system, inappropriate inflammatory responses are strongly implicated in the development and progression of cardiovascular and metabolic...
in increased liver expression levels of TNF-α and natural killer cell activity in splenocytes (12). However, the modulatory effect of oral NaNO2 (reported to suppress the mouse splenic immune tissues such as spleen and adipose tissue are not fully elucidated. On the other hand, there is considerable evidence from previous reports that long-term feeding with a high-fat diet (HFD) is associated with a state of low-grade chronic inflammation (13). It has been reported that choline-deficient high-fat diet (CDHFD) feeding for 8 wk leads to marked steatohepatitis but no fibrosis, but CDHFD feeding combined with intraperitoneal injection of NaNO2 (40 mg/kg/d) results in severe liver fibrosis (14). CDHFD feeding for 4 wk has no significant effects on hepatic gene expression of inflammatory cytokines, but CDHFD feeding combined with intraperitoneal injection of NaNO2 (40 mg/kg/d) results in increased liver expression levels of TNF-α and IL-1β (15). Based on these previous findings, we anticipated that HFD feeding in combination with NaNO2 consumption would augment inflammatory responses and tissue injury when compared with HFD feeding alone. In the present study, we first investigate effects of dietary NaNO2 supplementation on the expression of certain genes encoding pro- and anti-inflammatory cytokines and other inflammatory mediators in mouse immune tissues (i.e., spleen, abdominal adipose tissue, liver). To obtain more insights into the involvement of dietary NaNO2 in the pathogenesis of inflammatory diseases, we next compared effects of NaNO2 consumption versus HFD feeding, and elucidated their combined effects on the inflammatory gene expression profiles in mouse immune tissues.

**MATERIALS AND METHODS**

*Animal diets.* Casein, α-corn starch, sucrose, cellulose powder, AIN-76 mineral mixture (16), AIN-76 vitamin mixture (16), and choline bitartrate were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). DL-Methionine, soybean oil, lard, beef tallow and NaNO2 were obtained from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Purified standard low-fat diet (LFD) (5% fat, w/w) and high-fat diet (HFD) (21.5% fat, w/w) powders were prepared in our laboratory using food-grade ingredients. The composition of these diets is indicated in Table 1. NaNO2 was dissolved in boiling water. Diets containing NaNO2 were prepared by mixing 50 g of standard LFD powder and 100 g boiling water containing 30 or 120 mg NaNO2, or by mixing 50 g of HFD powder and 100 g boiling water containing 30 mg NaNO2. The final concentrations of NaNO2 in these diets were 0.02, 0.08 and 0.02% (w/w), respectively. Diets without NaNO2 were prepared by mixing 50 g standard LFD or HFD powder and 100 g boiling water. The test diets with or without NaNO2 were kept at 4°C until required.

**Animal maintenance and experimental design.** Specific-pathogen-free, 5-wk-old female BALB/c mice were obtained from Charles River Japan (Atsugi, Japan). These animals were maintained on a commercial laboratory chow (Oriental Yeast Co., Ltd.) and had access to water ad libitum. The non-purified diet comprised approximately 23.6% protein, 5.3% fat, 6.1% ash, 2.9% fiber and 54.4% nitrogen-free extracts. In Experiment I, after an acclimatization period (5 d), the mice were separated into three different dietary groups as follows: 1) standard LFD; 2) LFD with 0.02% NaNO2; and 3) LFD with 0.08% NaNO2. For Experiment II, after an acclimatization period (5 d), the mice were separated into four different dietary groups: 1) standard LFD; 2) LFD containing 0.02% NaNO2; 3) HFD; and 4) HFD containing 0.02% NaNO2. Before commencing with the test diets, the body weights were comparable among the animal groups. A 12-h/12-h light/dark cycle was maintained and the room temperature was kept at 23 ± 1°C in all groups. Food intake and body weights were monitored every other day and weekly, respectively. At the end of the dietary tests at 11 wk, the animals were sacrificed by decapitation between 8:30 and 10:30 a.m.

### Table 1. Composition of the purified powder diets used in this study.

|                      | Standard low-fat diet (LFD) (g/kg) | High-fat diet (HFD) (g/kg) |
|----------------------|-----------------------------------|---------------------------|
| α-Corn starch        | 550.75                            | 385.75                    |
| Sucrose              | 100                               | 100                       |
| Casein               | 200                               | 200                       |
| DL-Methionine        | 2.25                              | 2.25                      |
| Soybean oil          | 50                                | 50                        |
| Lard                 | 0                                 | 82.5                      |
| Beef tallow          | 0                                 | 82.5                      |
| Mineral mixture¹     | 35                                | 35                        |
| Vitamin mixture¹     | 10                                | 10                        |
| Choline bitartrate   | 2                                 | 2                         |
| Cellulose            | 50                                | 50                        |

¹ AIN-76 mineral and vitamin mixtures (16).
Blood was collected and allowed to clot for 1 h at room temperature. Serum was then separated by centrifugation at 1,200 \times g for 20 min at 4 °C and stored at −80 °C until analysis. Tissue samples were weighed and stored at −80°C or in RNAlater (Thermo Fisher Scientific, Tokyo, Japan) at −20°C. The experimental procedures used in the present study confirmed to the guidelines of the Animal Usage Committee of Sagami Women’s University (approval numbers: 2019-01, 2020-01).

Biochemical analyses of serum parameters. The serum nitrite/nitrate (NO\(_2^-)/\text{NO}_3^-\), ALT, triglyceride (TG), nonesterified fatty acids (NEFA) and total cholesterol levels were measured using commercially available kits as follows: Nitric oxide fluorometric assay kit (BioVision Inc., Milpitas, CA), transaminase CII-test, triglyceride E-test, nonesterified fatty acid C, cholesterol E-test (FUJIFILM Wako Pure Chemical Corporation) in accordance with the manufacturer’s instructions in each case. All assays were performed in duplicate and the data averages were statistically analyzed.

RNA extraction, cDNA synthesis and real-time RT-PCR. Total RNA was isolated from spleens, livers and abdominal adipose tissue (AT) of the mice with an Isogen according to the manufacturer’s instructions (Nippon Gene Co., Tokyo, Japan). The quantity and the quality of RNA samples were measured with a Nano Drop 1000 (Nanodrop Technologies, Wilmington, DE). cDNA synthesis was performed using random primers and a reverse transcription system (Promega, Madison, WI). Real-time quantitative RT-PCR analyses were performed in 20 μL reaction volumes using a KAPA SYBR FAST qPCR Kit (Nippon Genetics Co., Ltd., Tokyo, Japan) containing 12 pmol of primers and 35 ng of reverse transcribed total RNA. The resulting amplified products were analyzed using a Thermal Cycler Dice Real Time System TP870 (Takara Bio Inc., Tokyo, Japan). The primers used are listed in Table S1 (Supplemental Online Material). All PCR products were generated using a two-step protocol (95°C for 5 s, 60°C for 30 s) after an initial denaturation step (95°C for 30 s). The relative expression levels of the target gene products were calculated via the comparative threshold cycle method using Hprt1 as the normalization control.

### RESULTS

Food intake, total body, spleen, liver and abdominal AT weights, and serum levels of nitrite/nitrate and ALT in Experiment I

In the LFD-fed mice, the consumption of 0.02 and 0.08% NaNO\(_2\) had no significant effects on the total food intake within the experimental period (11 wk), or with the total body, liver and abdominal AT weights, or serum ALT levels (Table 2). NaNO\(_2\) consumption did however result in an increased spleen weight, which was greater in the LFD with 0.08% NaNO\(_2\)-fed mice. The daily intake of NaNO\(_2\) in the mice fed 533 g of the LFD with 0.02% NaNO\(_2\) and 538 g of the LFD with 0.08% NaNO\(_2\) for 11 wk were 1.38 and 5.59 mg/mouse/d, respectively. These animals thus consumed NaNO\(_2\) at a dosage of 61 and 250 mg/kg body weight/d, respectively, calculated from the average body weight over 11 wk (22.8 g for the LFD with 0.02% NaNO\(_2\)-fed mice and 22.4 g for the LFD with 0.08% NaNO\(_2\)-fed mice). Notably however, the serum nitrite+nitrate levels of the LFD with 0.08% NaNO\(_2\)-fed mice were only about 1.5-fold above those in the 0.02% NaNO\(_2\) group.

Effects of NaNO\(_2\) consumption on splenic expression of specific genes in Experiment I

The LFD with 0.08% NaNO\(_2\)-fed mice showed higher splenic mRNA levels of intracellular and cell surface markers for neutrophils (Ngp, NE, Ly6g, Mpo) and...
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Table 3. Effects of sodium nitrite (NaNO2) consumption in Experiment 1 on the splenic expressions of inflammatory and other specific genes.

| Gene                  | LFD1 | LFD +0.02% NaNO2 | LFD +0.08% NaNO2 | Relative mRNA level |
|-----------------------|------|------------------|------------------|--------------------|

Neutrophil markers

- Nggp 1.0±0.36b 2.03±0.51a 2.53±0.40b
- Ne 1.0±0.33b 2.13±0.71b 4.35±0.99b
- Ly6g 1.0±0.26b 1.66±0.35b 4.12±0.82a
- Mpo 1.0±0.29b 2.11±0.67b 4.87±1.58a

Eosinophil markers

- Epo 1.0±0.22a 1.90±0.55b 4.54±0.78a
- Ear6 1.0±0.34a 1.65±0.48b 3.86±0.52a

S100 family members

- S100AA8 1.0±0.38a 1.62±0.41b 3.39±0.59a
- S100A9 1.0±0.35a 1.36±0.29b 2.95±0.53a

Macrophage markers

- Emr1 1.0±0.12a 0.74±0.05a 0.77±0.07b
- Itgax 1.0±0.10a 0.78±0.08b 0.67±0.11b
- CD68 1.0±0.07a 0.78±0.08b 0.78±0.03b
- CD206 1.0±0.14a 0.71±0.08b 0.72±0.07b
- Cox2 1.0±0.08a 0.81±0.09b 0.76±0.03b
- Tlr4 1.0±0.09a 0.73±0.07b 0.79±0.02b
- Tlr5 1.0±0.28a 0.40±0.14b 0.52±0.10a
- Tlr6 1.0±0.06a 0.63±0.01c 0.70±0.04b
- Tlr7 1.0±0.15a 0.55±0.03c 0.71±0.05b

T-cell markers

- Cd3 1.0±0.22a 0.66±0.05b 0.63±0.08b
- Cd4 1.0±0.20a 0.77±0.06b 0.62±0.05b

NK-cell marker

- Dc56 1.0±0.22a 0.73±0.06b 0.74±0.06b

B-cell markers

- Cd20 1.0±0.11a 0.82±0.07b 0.66±0.06c
- Cd40 1.0±0.08a 0.78±0.05b 0.77±0.07b

Cytokines

- Tnf-α 1.0±0.23a 0.73±0.07b 0.65±0.15b
- Il-6 1.0±0.11a 0.71±0.11b 0.76±0.09b
- Il-1β 1.0±0.23a 0.73±0.13b 0.62±0.04b
- Ifn-γ 1.0±0.20a 0.74±0.04b 0.65±0.08b
- Il-18 1.0±0.11a 0.72±0.02b 0.72±0.05b
- Il-10 1.0±0.13a 0.59±0.04b 0.62±0.04b

Interleukin receptor antagonists

- Il-1ra 1.0±0.13a 0.78±0.08b 0.76±0.08b
- Il-6ra 1.0±0.14a 0.65±0.06b 0.70±0.04b

Cell adhesion molecules

- Icam-1 1.0±0.11a 0.74±0.12b 0.71±0.05b
- Vcam-1 1.0±0.12a 0.68±0.08b 0.69±0.04b

Metallothionein

- Mt2 1.0±0.33b 1.94±0.44a 2.37±0.36a

Cell proliferation marker

- Mki67 1.0±0.24a 1.69±0.27a 1.60±0.16a

Genes associated with cell cycle progression

- Cyclin B 1.0±0.26b 1.65±0.26a 1.68±0.22a
- Cdc2 1.0±0.15a 1.60±0.20a 1.68±0.31a
- Cdc3 1.0±0.20b 1.67±0.22a 1.41±0.11b

Members of the cytochrome P450 family

- Cyp4v3 1.0±0.13a 0.72±0.05b 0.63±0.05b
- Cyp2d22 1.0±0.25a 0.56±0.07b 0.61±0.05b
- Cyp27a1 1.0±0.04c 0.65±0.05b 0.69±0.04b

1 LFD: Standard low-fat diet. Each value represents a mean±standard deviation (n=7/test group). The mean values highlighted with different superscripts (a–c) for the same gene are significantly different (p<0.05).

eosinophils (Epo, Ear6), in addition to S100 family members (S100A8, S100A9), Ca2+ binding proteins that are constitutively expressed in neutrophils and monocytes (17) (Table 3). The LFD with 0.02% NaNO2-fed mice also exhibited higher splenic mRNA levels for some of these genes (Ngg, Ne, Epo, Ear6, S100A8), but at a lower level than the 0.08% NaNO2 group. In contrast, both NaNO2 groups showed comparably lower splenic mRNA levels for cell markers of macrophages (Emr1, Itgax, Cd68, Cd206, Cox2, Tlr4, Tlr5, Tlr6, Tlr7), T- (Cd3, Cd4), NK- (Cd56) and B-cells (Cd20, Cd40), pro- and anti-inflammatory cytokines (Tnf-α, Il-6, Il-1β, Ifn-γ, Il-18, Il-10), interleukin receptor antagonists (Il1ra, Il6ra) and cell adhesion molecules (Icam-1, Vcam-1). The consumption of 0.02 and 0.08% NaNO2 both caused elevated splenic mRNA levels for a member of the metallothioneins (Mt2), which play important roles in metal ion homeostasis and detoxification (18), a key cell proliferation marker (Mki67), and genes associated with progression through the cell cycle (Cyclin B, Cdc20, Cdc3). The splenic mRNA levels for some members of the cytochrome P450 family (Cyp4v3, Cyp2d22, Cyp27a1) were found to be decreased in the 0.02 and 0.08% NaNO2 groups. However, the NaNO2 diets had no significant effects on the splenic mRNA levels for genes encoding key enzymes of the heme and porphyrin biosynthesis pathway (Fech, Pcox, Uros) (data not shown).

Effects of NaNO2 consumption on specific genes in the liver and abdominal AT in Experiment I

Both of the NaNO2 dietary groups showed slightly higher Tnf-α mRNA levels (mean±SD 1.00±0.19 for the LFD, 1.25±0.16 for the LFD with 0.02% NaNO2, 1.24±0.19 for the LFD with 0.08% NaNO2; n=7/test group) and lower Il1ra mRNA levels (1.00±0.21 for the LFD, 0.69±0.16 for the LFD with 0.02% NaNO2, 0.77±0.08 for the LFD with 0.08% NaNO2) in the liver. The consumption of 0.02% and 0.08% NaNO2 also caused increased in the liver mRNA levels for a neutrophil marker (Ne) (1.00±0.25 for the LFD, 1.45±0.38 for the LFD with 0.02% NaNO2, 1.50±0.24 for the LFD with 0.08% NaNO2), and the 0.08% NaNO2 consumption led to elevated liver Mpo mRNA levels (1.00±0.43 for the LFD, 1.79±0.43 for the LFD with 0.02% NaNO2, 2.70±1.10 for the LFD with 0.08% NaNO2). There were no significant effects at either NaNO2 concentration on liver mRNA levels for other immune cell markers and inflammatory cytokines, C-reactive protein (Crp, a biomarker of the inflammatory response), and other specific genes such as metallothionein (Mt2), cell proliferation marker (Mki67), genes associated cell cycle progression (Cyclin B, Cdc20, Cdc3), or members of the cytochrome P450 family (Cyp4v3, Cyp2d22, Cyp27a1) (data not shown). No significant effects of NaNO2 on these specific genes were found in the abdominal AT (data not shown).

Food intake and body, spleen, liver, and abdominal AT weight effects in Experiment II

HFD feeding caused a decrease in the total food intake over the experimental period of 11 wk compared with...
Effects of NaNO2 consumption and HFD feeding on the

Table 4. Effects of sodium nitrite (NaNO2) consumption and high-fat diet (HFD) feeding in Experiment II on food intake, body, spleen, liver and abdominal adipose tissue (AT) weights, and serum levels of nitrite+nitrate, alanine aminotransferase (ALT), triglyceride (TG), nonesterified fatty acid (NEFA) and total cholesterol.

|                          | LFD1                                       | LFD +0.02% NaNO2 | HFD +0.02% NaNO2 | Two-way ANOVA p<0.05 |
|--------------------------|--------------------------------------------|------------------|------------------|---------------------|
| Total food intake (g/mouse/11 wk) | 557±13                                    | 539±2            | 502±22           | 495±16 HFD          |
| Body weight (g)          | 21.6±2.1b                                  | 23.7±1.0b        | 27.4±2.5a        | 26.6±2.2a HFD, Interaction NaNO2, HFD |
| Spleen weight (mg)       | 103±19b                                    | 125±13a          | 126±15a          | 133±7a HFD, Interaction NaNO2 |
| Liver weight (g)         | 0.97±0.07                                  | 1.00±0.09        | 1.30±0.13        | 1.22±0.11 HFD       |
| Abdominal AT weight (g)  | 0.41±0.18                                  | 0.50±0.09        | 0.78±0.25        | 0.78±0.33 HFD       |
| Serum nitrite+nitrate level (μmol/L serum) | 12.6±4.7                                  | 36.6±13.9        | 9.4±3.5          | 46.9±11.6 NaNO2     |
| Serum ALT level (IU/L serum) | 9.6±4.1                                   | 6.0±2.4          | 6.8±2.9          | 7.6±2.2 HFD         |
| Serum TG level (mg/dL serum) | 80.0±16.0                                 | 89.3±23.8        | 147.0±50.3       | 143.4±44.6 HFD      |
| Serum NEFA level (mEq/L serum) | 0.97±0.16                                 | 0.85±0.18        | 0.99±0.16        | 0.85±0.20 NaNO2     |
| Serum total cholesterol level (mg/dL serum) | 103.6±15.4                                | 104.2±16.2       | 148.6±44.2       | 132.9±16.8 HFD      |

1 LFD: Standard low-fat diet. ANOVA: analysis of variance. Each value represents a mean±standard deviation (n=10/test group). The mean values highlighted with different superscripts (a, b) for the same parameter are significantly different (p<0.05).

LFD feeding (Student t test, p<0.05), but NaNO2 consumption had no significant effects on this (Table 4). The HFD with or without NaNO2-fed mice showed increased body weights compared with their LFD counterparts. The LFD with NaNO2 and the HFD with and without NaNO2 resulted in higher serum nitrite levels compared with the LFD without NaNO2. HFD feeding resulted in increased weights of the liver and abdominal AT compared with LFD feeding (Student t test, p<0.05), but NaNO2 supplementation had no significant effects on these weights.

Serum levels of nitrite/nitrate, ALT, TG, NEFA and total cholesterol in Experiment II

The daily intake of NaNO2 in the mice fed 539 g of the LFD with 0.02% NaNO2 and 495 g of the HFD with 0.02% NaNO2 for 11 wk were 1.40 and 1.29 mg/mouse/d, respectively. These animals thus consumed NaNO2 at a dosage of 63 and 55 mg/kg body weight/d, respectively, calculated from their average body weights over 11 wk (22.2 g for the LFD with 0.02% NaNO2-fed mice and 23.4 g for the HFD with 0.02% NaNO2-fed mice). NaNO2 consumption resulted in increased serum nitrite+nitrate levels (Student t test, p<0.05). NaNO2 consumption and HFD feeding were each observed not to significantly affect serum ALT levels. HFD feeding resulted in increased serum levels of TG and total cholesterol compared with LFD feeding (Student t test, p<0.05). NaNO2 consumption led to a decrease in the serum NEFA levels (Student t test, p<0.05).

Effects of NaNO2 consumption and HFD feeding on the splenic expression of specific genes in Experiment II

NaNO2 consumption resulted in increases to the splenic mRNA levels for cell markers of neutrophils (Ngp, NE, Ly6g, Mpo) and eosinophils (Epo, Ear6), and S100 family members (S100A8, S100A9) compared with no consumption (Student t test, p<0.05; Fig. 1). HFD feeding also caused elevated splenic mRNA levels for these cell markers for neutrophils and eosinophils, and S100A8 compared with LFD feeding (Student t test, p<0.05). The HFD with NaNO2-fed mice showed higher splenic mRNA levels for Ngp compared with the LFD with and without NaNO2-fed and the HFD without NaNO2-fed mice. The LFD with NaNO2- and the HFD with and without NaNO2-fed mice exhibited lower splenic mRNA levels for intracellular and cell surface markers of macrophages (Emr1, Itgax, CD68, CD206, Dectin-1, TLR4, TLR6, TLR7), T-cells (CD3, CD4), NK-cell (CD56), and B-cells (CD20, CD40) when compared with the LFD without NaNO2-fed mice (Fig. 2). The LFD with NaNO2- and the HFD with and without NaNO2-fed mice showed lower splenic mRNA levels for pro- and anti-inflammatory cytokines (TNF-α, IL-6, IFN-γ, IL-18, IL-10, TGF-β), interleukin receptor antagonists (IL1ra, IL6ra) and cell adhesion molecules (ICAM-1, VCAM-1) when compared with the LFD without NaNO2-fed mice (Fig. 3).

The LFD with NaNO2- and the HFD with and without NaNO2-fed mice exhibited higher mRNA levels for a member of the metallothioneins (Mtr2), a key cell proliferation marker (Mki67), and genes associated with progression through the cell cycle (Cyclin B, Cdc20, Cdc63) compared with the LFD without NaNO2-fed mice (Fig. 4). In contrast, the LFD with NaNO2- and the HFD with and without NaNO2-fed mice showed lower splenic mRNA levels for genes encoding some members of the cytochrome P450 family (Cyp4v3, Cyp4v3, Cyp2d22, Cyp27a1) compared with the LFD without NaNO2-fed mice. Neither NaNO2 consumption nor HFD feeding was observed to significantly affect the splenic mRNA levels for key enzymes of the heme and porphyrin biosynthesis path-
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Fig. 1. Effects of dietary sodium nitrite (NaNO₂) consumption and high-fat diet (HFD) feeding on the splenic mRNA levels for intracellular and cell surface markers of neutrophils (A, Ngp; B, NE; C, Ly6g; D, Mpo) and eosinophils (E, Epo; F, Ear6), and of S100 family members (G, S100A8; H, S100A9). The transcript levels for each gene are expressed as relative mRNA levels normalized to Hprt1. Each value represents the mean±standard deviation (n=10/test group). Significant differences were assessed by two-way ANOVA. The mean values highlighted with different superscripts (a, b) were significantly different (p<0.05).
Effects of NaNO₂ consumption and HFD feeding on expression of specific genes in the liver and abdominal AT in Experiment II

NaNO₂ consumption and HFD feeding each resulted in increased liver mRNA levels for neutrophil markers (Mpo, NE) (Student t test, p<0.05) (Table 5). The LFD with NaNO₂-, and the HFD with and without NaNO₂-fed mice showed higher mRNA levels for a macrophage intracellular marker (COX2) compared with the LFD without NaNO₂-fed mice. NaNO₂ consumption and HFD feeding each resulted in increased mRNA levels for a B
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Cell marker (CD20) and pro-inflammatory cytokines (TNF-α, IL-18) (Student t test, p < 0.05). The LFD with NaNO2- and the HFD with and without NaNO2-fed mice exhibited lower liver mRNA levels for interleukin receptor antagonists (IL1ra, IL6ra) compared with the LFD without NaNO2-fed mice. HFD feeding, but not NaNO2 consumption, caused a slight increase in the liver mRNA levels for a biomarker of the inflammatory response (CRP) (Student t test, p < 0.05). In terms of liver factors, neither NaNO2 consumption nor HFD feeding had significant effects on the mRNA levels for other inflammatory and specific genes (data not shown). With regard to the abdominal AT, NaNO2 consumption and HFD feeding had no significant effects on the aforementioned specific genes (data not shown).

**DISCUSSION**

Neutrophil-driven inflammation is recognized as a common mechanism underlying many pathological conditions, including atherosclerosis, cardiovascular and autoimmune diseases (19). Neutrophils utilize granule proteins to orchestrate the inflammatory response, and eosinophil-derived cationic granule protein participates in generating reactive oxidants and radical species (20). It has been reported that dietary NaNO2 consumption (100 mg/kg body weight/d) for 21 d results in an increase in the percentage of neutrophils and a decrease in percentage of lymphocytes in the mouse spleen (12). In our present study, splenic mRNA levels for cell markers of neutrophils and eosinophils were found to be upregulated, while those of macrophages, T-, NK- and B-cells were downregulated, by NaNO2 consumption. Similar results were also observed by HFD feeding. These suggested that NaNO2 consumption and HFD feeding can each induce an increase in proportion of neutrophils and eosinophils, and a
Fig. 3. Effects of dietary sodium nitrite (NaNO₂) consumption and high-fat diet (HFD) feeding on the splenic mRNA levels for pro- and anti-inflammatory cytokines (A, TNF-α; B, IL-6; C, IL-1β; D, IFN-γ; E, IL-18; F, IL-10; G, TGF-β), interleukin receptor antagonists (H, IL1ra; I, IL6ra) and cell adhesion molecules (J, ICAM-1; K, VCAM-1). The transcript levels for each gene are expressed as relative mRNA levels normalized to Hprt1. Each value represents the mean ± standard deviation (n=10/test group). Significant differences were assessed by two-way ANOVA. The mean values highlighted with different superscripts (a, b) were significantly different (p<0.05).
Fig. 4. Effects of dietary sodium nitrite (NaNO₂) consumption and high-fat diet (HFD) feeding on the splenic mRNA levels for the metallothionein Mt2 (A), the key cell proliferation marker Mki67 (B), genes associated with progression through the cell cycle (C–E, Cyclin B, Cdc20, and Cdc3, respectively) and members of the cytochrome P450 family (F–H, Cyp4v3, Cyp2d22, and Cyp27a1, respectively). The transcript levels for each gene are expressed as relative mRNA levels normalized to Hprt1. Each value represents the mean±standard deviation (n=10/test group). Significant differences were assessed by two-way ANOVA. The mean values highlighted with different superscripts (a–c) were significantly different (p<0.05).
Table 5. Effects of sodium nitrite (NaNO₂) consumption and high-fat diet (HFD) feeding in Experiment II on the expression of liver-specific genes.

| Gene                        | LFD¹ | LFD +0.02% NaNO₂ | HFD | HFD +0.02% NaNO₂ | Two-way ANOVA p<0.05 |
|-----------------------------|------|------------------|-----|------------------|---------------------|
| Neutrophil markers          |      |                  |     |                  |                     |
| Mpo                         | 1.00±0.35 | 1.71±0.48 | 1.41±0.32 | 2.21±0.92 | NaNO₂, HFD         |
| NE                          | 1.00±0.50 | 1.50±0.54 | 1.63±0.36 | 2.45±1.44 | NaNO₂, HFD         |
| Macrophage marker           |      |                  |     |                  |                     |
| COX2                        | 1.00±0.61⁻ | 1.46±0.43ᵇ | 2.30±0.68ᵃ | 1.73±0.42ᵇ | HFD, Interaction    |
| B cell marker               |      |                  |     |                  |                     |
| CD20                        | 1.00±0.37 | 1.54±0.45 | 1.56±0.31 | 1.67±0.30 | NaNO₂, HFD         |
| Cytokines                   |      |                  |     |                  |                     |
| TNF-α                       | 1.00±0.21 | 1.28±0.27 | 1.19±0.55 | 1.65±0.46 | NaNO₂, HFD         |
| IL-18                       | 1.00±0.32 | 1.58±0.25 | 1.48±0.20 | 1.78±0.32 | NaNO₂, HFD         |
| Interleukin receptor antagonists |  |  |  |  | |
| IL1ra                       | 1.00±0.21ᵃ | 0.72±0.24ᵇ | 0.62±0.15ᵇ | 0.63±0.14ᵇ | NaNO₂, HFD, Interaction |
| IL6ra                       | 1.00±0.10ᵃ | 0.79±0.23ᵇ | 0.52±0.09ᶜ | 0.60±0.12ᶜ | HFD, Interaction    |
| Biomarker of the inflammatory response |  |  |  |  |  |
| CRP                         | 1.00±0.13 | 0.98±0.11 | 1.08±0.14 | 1.24±0.31 | HFD                 |

¹LFD: Standard low-fat diet. ANOVA: analysis of variance. Each value represents a mean±standard deviation (n=10/group). The mean values highlighted in the same row with different superscripts (a–c) are significantly different (p<0.05).

decrease in proportion of macrophages, T-, NK- and B-cells in the spleen. It has been reported that a HFD that is rich in polyunsaturated fatty acids, but not in saturated fatty acids, causes the enhanced recruitment of neutrophils from the circulation into the spleen and thereby results in a delay in neutrophil cell death in the spleen (21). In our present study however, the HFD powder we used consisted of 8.25% (w/w) lard and 8.25% beef tallow, which are rich in saturated fatty acids, in addition to 5% soybean oil (Table 1). Therefore, it has been suggested that a HFD that is rich in saturated fatty acids can also induce an increase in the proportion of neutrophils and eosinophils in the spleen. In Experiment I in our current study, an LFD supplemented with 0.08% NaNO₂ produced a greater increase in the splenic mRNA levels for neutrophil and eosinophil markers compared with the LFD with 0.02% NaNO₂, but there was no significant difference in most of the transcript levels for cell markers of macrophages, T-, NK- and B-cells between these groups. It remains unclear why the consumption of 0.08% NaNO₂ induced no further decreases in the splenic mRNA levels for cell markers of macrophages, T-, NK- and B-cells.

Lamas et al. (22) have reported that a fat-rich hypercaloric diet for 5 wk in rats resulted in a downregulation of the splenic mRNA levels for pro-inflammatory cytokines (TNF-α, IL-6) compared with the animals on a standard chow diet. Another study found that HFD feeding (60% kcal from fat) for 8 wk decreases the mouse splenic protein levels of both pro- and anti-inflammatory cytokines (IL-1β, IL-10) compared with a standard chow diet (23). In our current study, NaNO₂ consumption and HFD feeding each resulted in decreased splenic mRNA levels for pro- and anti-inflammatory cytokines (TNF-α, IL-6, IL-1β, IFN-γ, IL-18, IL-10, TGF-β) and interleukin receptor antagonists (IL1ra, IL6ra). It is well known that macrophages produce proinflammatory cytokines such as TNF-α, IFN-γ, IL-1β and IL-18 (24). Pestka et al. (25) have demonstrated that activated B-cells, which mature in the marginal zone of the spleen, produce large amounts of IL-10. Thus, we speculate from our current findings that the NaNO₂ consumption- or HFD feeding-induced reduction of splenic mRNA levels for pro- and anti-inflammatory cytokines and interleukin receptor antagonists may, at least in part, reflect a decreased proportion of macrophages and B cells in the spleen.

Compared with the LFD without NaNO₂-fed control mice, the HFD with NaNO₂-fed mice showed elevated serum nitrite+nitrate levels, as well as increased body weight (Table 4). Therefore, based on our current findings, we anticipated that dietary NaNO₂ in combination with a HFD would augment the observed reductions in the splenic mRNA levels for cell markers of macrophages, T-, NK- and B-cells, pro- and anti-inflammatory cytokines, and interleukin receptor antagonists. Contrary to our expectations however, no further reductions in the splenic transcripts for these genes were induced by this dietary combination. A mechanistic explanation for this remains to be elucidated. On the other hand, dietary NaNO₂ combined with HFD feeding caused a more profound increase in the splenic mRNA levels for a neutrophil marker (Ngp) when compared with dietary NaNO₂ alone (Fig. 1A). However, there was no significant interaction in the splenic mRNA levels for other markers of neutrophils and eosinophils between NaNO₂ consumption and HFD feeding (Fig. 1B-F). Thus, their combined effects on the splenic transcripts
for these genes could not be assessed. IL-10 has a central role in infection by limiting the immune response to pathogens and thereby preventing damage to the host (26). It has been reported that large amounts of IL-10 produced by activated splenic B cells play a regulatory role in suppressing harmful immune responses (25). Gotth et al. (23) have found that HFD-induced obesity causes a reduced splenic IL-10 production and a decrease in the serum IL-10 levels, and further demonstrated that these phenomena are responsible for the development of non-alcoholic fatty pancreas disease that is contributed to by inflammation.

In our present analyses in the mouse, NaNO2 consumption as well as HFD feeding resulted in a decrease in the splenic IL-10 mRNA levels. To clarify the effects of the NaNO2 consumption and HFD feeding-induced reduction of splenic mRNA levels for pro- and anti-inflammatory cytokines on the corresponding protein levels, we measured the splenic and serum protein levels of TNF-α, IL-6, IFN-γ and IL-10 using an enzyme-linked immunosorbet assay. However, these cytokine levels in a 10% (w/v) spleen homogenate and serum diluted 2-fold with assay diluent were below detectable levels (TNF-α: 3.125 pg/mL sample, IL-10: <31 pg/mL sample, IFN-γ: <2.74 pg/mL sample, IL-6: <0.82 pg/mL sample) in all four test groups in Experiment II. Thus, we could not confirm reduced protein levels in the spleen and serum. Further studies are needed to assess the involvement of the NaNO2-induced reduction of splenic IL-10 mRNA levels on inflammatory responses.

HFD-induced obesity stimulates the AT release of inflammatory cytokines such as TNF-α and IL-6, and the enhanced IL-6 concentration then stimulates the liver to synthesize and secrete C-reactive protein (27). It has been reported that consumption of a HFD containing 45% fat from lard for 24 wk results in elevated AT mRNA levels for pro-inflammatory genes (TNF-α, IL-1β, Mcp-1, Emr1) compared to LFD feeding (28). HFD (60% kcal from fat) feeding for 16 wk was reported in another study to lead to increases in the liver TNF-α and IL-1 mRNA levels compared with LFD feeding (29). In contrast, we found in our present study that the HFD feeding resulted in only a slight increase in the liver TNF-α mRNA levels but no increase in mRNA levels for liver IL-1β or abdominal AT TNF-α and IL-1β. These more marginal effects we observed in our present analyses may be attributable to our study design (i.e. the 21.5% fat test diet and 11-wk experimental period).

The upregulation of the transcript levels for splenic metallothionein (Mt2), which protects blood cells from oxidative stress-induced cytotoxicity and genotoxicity, was observed in our experimental mice following NaNO2 consumption or HFD feeding and likely reflects increased oxidative stress. The LFD with NaNO2-, and the HFD with and without NaNO2-fed mice showed higher splenic mRNA levels for the Mk67 proliferation marker and for genes associated with cell cycle progression (Cyclin B, Cdc20, Cdc6a3) compared with the LFD without NaNO2-fed mice. This indicated that cellular proliferation is enhanced in the spleen by NaNO2 consumption or HFD feeding. In contrast, the downregulation of splenic mRNA levels for some members of the cytochrome P450 family (Cyp4v3, Cyp2d22, Cyp27a1), which are associated with heme and iron binding, or monoxygenase activity, was observed following NaNO2 consumption or HFD feeding, and likely influences the splenic cytochrome P450 activity.

The spleen is the largest lymphoid organ in the body and plays an important role in host immune functions. Our present results indicate that the spleen is one of the target tissues for dietary NaNO2, the modulatory effects of which are similar to those of HFD feeding in relation to splenic transcripts for immune and inflammatory genes. Notably however, dietary NaNO2 combined with HFD feeding shows no additive effect in terms of the gene modulation compared with dietary NaNO2 alone. Because high amounts of dietary NaNO2 were used in the animal model system described in the present study, it remains to be determined whether a normal human daily intake of NaNO2 would exhibit, in humans, similar effects to those shown here.

Authorship
MO performed the experiments and co-wrote the manuscript. YO performed the experiments. KH, KS, NT and YT performed the analyses and interpreted the data. TN co-wrote the manuscript.

Disclosure of state of COI
No conflicts of interest to be declared.

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Supporting information
Supplemental Online Material is available on J-STAGE.

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