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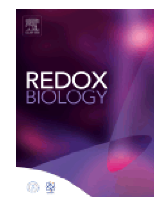

Research paper

# TEMPOL increases $NAD^+$ and improves redox imbalance in obese mice

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Continuous energy conversion is controlled by reduction–oxidation (redox) processes. $NAD^+$ and NADH represent an important redox couple in energy metabolism. 4-Hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL) is a redox-cycling nitroxide that promotes the scavenging of several reactive oxygen species (ROS) and is reduced to hydroxylamine by NADH. TEMPOL is also involved in $NAD^+$ production in the ascorbic acid–glutathione redox cycle. We utilized the chemical properties of TEMPOL to investigate the effects of antioxidants and $NAD^+$/NADH modulators on the metabolic imbalance in obese mice. Increases in the $NAD^+$/NADH ratio by TEMPOL ameliorated the metabolic imbalance when combined with a dietary intervention, changing from a high-fat diet to a normal diet. Plasma levels of the superoxide marker dihydroethidium were higher in mice receiving the dietary intervention compared with a control diet, but were normalized with TEMPOL consumption. These findings provide novel insights into redox regulation in obesity.

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## 1. Introduction

The worldwide prevalence of obesity is increasing each year. Obesity is associated with the risk of death from all cancers [1], shortened lifespan and decrease of healthy years [2]. However, effective therapeutic strategies for obesity are not known.

Obesity is a condition of increased adipose tissue mass, causing functional abnormalities in energy metabolism. Continuous energy conversion is tightly controlled by reduction–oxidation (redox) processes. $NAD^+$ and NADH represent an important redox couple for energy metabolism [3]. Over the last decade, the role of $NAD^+$ has become increasingly understood with discovery of proteins, such as sirtuins, that consume $NAD^+$ and function as metabolic regulators [4]. In recent reports, regulation of the $NAD^+$/NADH ratio led to increased energy metabolism [3]. Strategies for such regulation include: activation of related enzymes such as AMP-activated protein kinase [5] and nicotinamide phosphoribosyl transferase [6]; providing precursors for $NAD^+$ *de novo* and salvage pathways [7,8]; modulation of enzymes that consume $NAD^+$ such as poly(ADP-ribose)polymerase and CD38 [9,10]; and providing substrates of NADH:quinone oxidoreductase [11]. These studies showed that increasing the $NAD^+$/NADH ratio, including by promoting NADH oxidation, is critical to accelerating energy metabolism. In obese mice, the $NAD^+$/NADH ratio was lower than in lean mice [12], further suggesting that increasing the $NAD^+$/NADH ratio would be necessary for changing metabolic state to maintain homeostasis.

In contrast, in adipose tissue and plasma, oxidative stress and generation of reactive oxygen species (ROS) is greater in obese mice than in lean mice [13]. To decrease oxidative stress and its resulting tissue damage, administration of antioxidants has been considered [14].

Both approaches have been shown to attenuate disease symptoms, yet they are completely contradictory. While one involves increasing oxidation from NADH to $NAD^+$ the other is a process involving reduction, for example, to decrease ROS levels. We hypothesized that obesity leads to a growing imbalance between reduction–oxidation (redox) status in the body and that correction of this imbalance would be beneficial.

Here, we proposed a strategy to increase the $NAD^+$/NADH ratio and decrease ROS production. To regulate redox status in the body, we focused on a small redox-cycling nitroxide antioxidant, the 4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL). This

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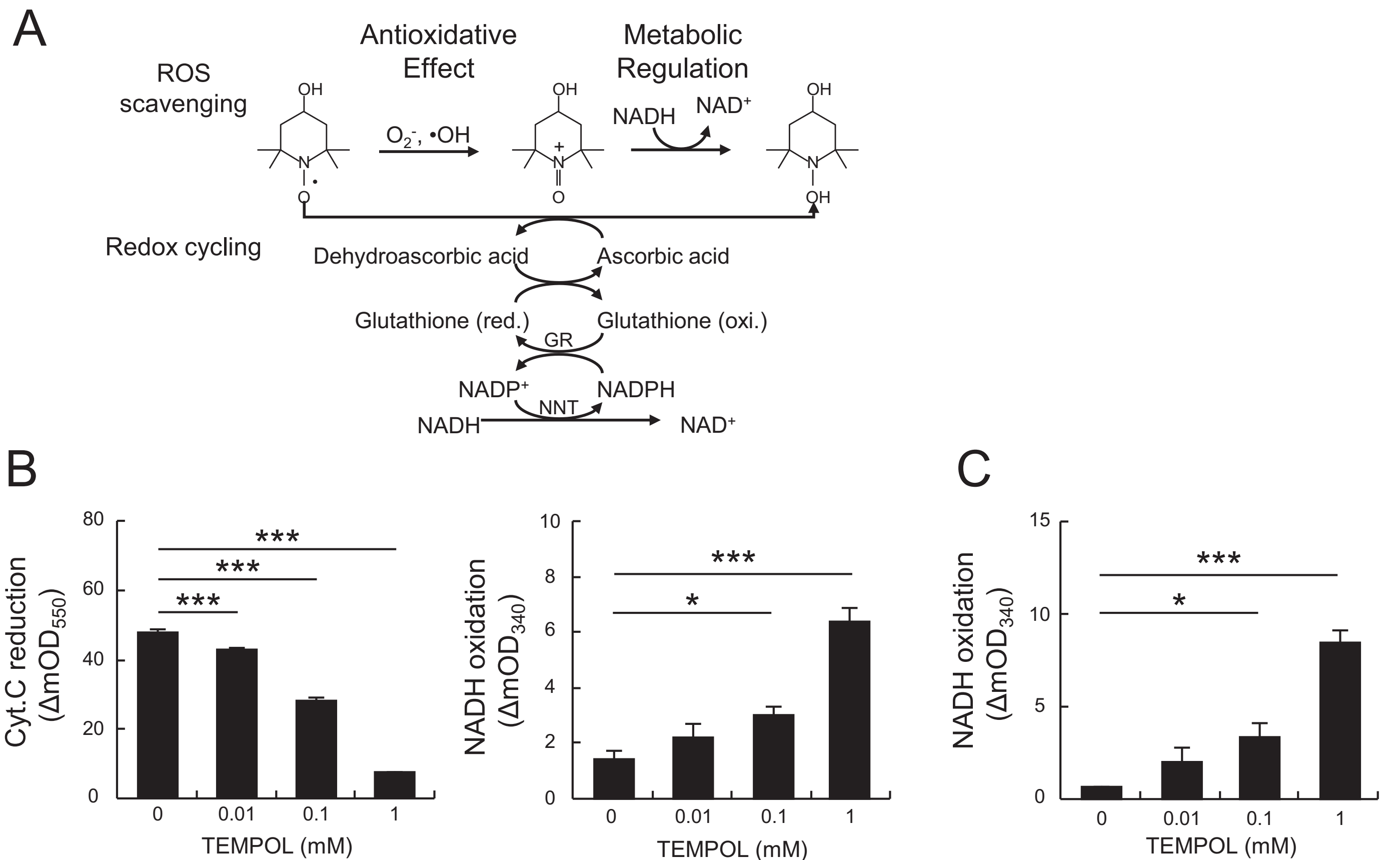


**Fig. 1.** TEMPOL increased the $NAD^+$/NADH ratio via ROS scavenging and the redox cycling system *in vitro*. (A) Schematic illustration of two pathways of $NAD^+$ generation by TEMPOL. GR, glutathione reductase; NNT, nicotinamide nucleotide transhydrogenase. (B) Superoxide scavenging and simultaneous $NAD^+$ production in the hypoxanthine–xanthine oxidase system. The reaction with superoxide was measured by the rate of reduction of ferricytochrome c at 550 nm. Reaction mixtures contained 120 μM hypoxanthine, 0.005 units xanthine oxidase, 24 μM cytochrome c and 1 mM NADH in 10 mM phosphate buffer (pH 7.4) with TEMPOL (0–1 mM, as indicated in the figure). (**C**) $NAD^+$ production in the AsA–GSH redox cycle. NADH oxidation was measured at 340 nm. The reaction mixture contained 1 mM ascorbic acid (AsA), 1 mM glutathione (GSH), 1 unit of GR and 0.5 mM NADH in 10 mM phosphate buffer (pH 7.4) with TEMPOL (0–1 mM). Values are means ± SD (n=4). $^{*}p<0.05$ and $^{***}p<0.005$ compared with 0 mM.

molecule has been reported to have two redox potential [15]. In the oxidation process, an oxoammonium cation was generated by the diffusion-controlled reaction of superoxide or hydroxyl radical with the aminoxyl group of TEMPOL, and then was reduced by NADH to the hydroxylamine [15,16]. During these reactions, TEMPOL can both reduce ROS and oxidize NADH to $NAD^+$. In the reduction process, TEMPOL was directly reduced to the corresponding hydroxylamine by ascorbic acid, resulting in formation of the oxidation product dehydroascorbic acid (DHA) [17]. In this case, DHA can increase the $NAD^+$ concentration via a redox cycling system comprised of glutathione (GSH), NADPH and NADH [18]. We therefore assumed that TEMPOL could indirectly generate $NAD^+$ by an NADH oxidation process via two different pathways, involving either ROS scavenging or the AsA–GSH redox cycling system (Fig. 1A).

In our study, we employed TEMPOL to investigate modulation of the $NAD^+$/NADH ratio to address metabolic imbalances in obese mice. We examined effects of TEMPOL in mice under dietary intervention following a high-fat diet (HFD), to assess dietary impacts on its effectiveness.

## 2. Materials and methods

### 2.1. Materials

4-Hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl (TEMPOL) was from Sigma-Aldrich Japan (Tokyo, Japan). Xanthine oxidase, glutathione reductase (GR), $NAD^+$, NADH, alcohol dehydrogenase from yeast and malate dehydrogenase from yeast were from Oriental Yeast Co., Ltd (Tokyo, Japan). The following were from Wako Pure Chemical Industries (Osaka, Japan): ferricytochrome c (from horse heart), hypoxanthine, cholesterol assay kit (Cholesterol E test), triglyceride assay kit (Triglyceride E test), Tiron, ascorbic acid, and glutathione. Dihydroethidium (DHE) was purchased from Life Technologies Japan Ltd. (Tokyo, Japan).

### 2.2. In vitro chemical reaction of TEMPOL

Superoxide was generated by the aerobic reaction of hypoxanthine and xanthine oxidase in the presence of ferricytochrome c and NADH. To estimate its reaction with superoxide, the rate of reduction of ferricytochrome c was measured at 550 nm using a grating-based spectrometer (SH-1000 Lab; Corona Electric Co., Ltd., Tokyo, Japan). This machine was equipped with a thermostat-controlled cell for the reaction mixture, which contained 120 μM hypoxanthine, 0.005 units xanthine oxidase, 24 μM cytochrome c and 1 mM NADH in 10 mM phosphate buffer (pH 7.4) with TEMPOL (0–1 mM, as indicated).

NADH oxidation was measured at 340 nm to assess effects of TEMPOL in a redox cycling system. The reaction mixture contained 1 mM ascorbic acid (AsA), 1 mM glutathione (GSH), 1 unit GR and 0.5 mM NADH in 10 mM phosphate buffer (pH 7.4) with TEMPOL (0–1 mM).

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