The nitroxide radical TEMPOL prevents obesity, hyperlipidaemia, elevation of inflammatory cytokines, and modulates atherosclerotic plaque composition in apoE<sup>−/−</sup> mice

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High-fat diet

High-fat diet plus TEMPOL in food

Atherosclerosis
Obesity
Hyperlipidemia
Elevated inflammatory markers

Decreased obesity
Decreased lipid levels
More stable lesion phenotype
Normalised levels of IL-6, MCP-1, myeloperoxidase, serum amyloid A, adiponectin, leptin
ABSTRACT

Objective: The nitroxide compound TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl radical) has been shown to prevent obesity-induced changes in adipokines in cell and animal systems. In this study we investigated whether supplementation with TEMPOL inhibits inflammation and atherosclerosis in apoE−/− mice fed a high fat diet (HFD).

Methods: ApoE−/− mice were fed for 12 weeks on standard chow diet or a high-fat diet. Half the mice were supplemented with 10 mg/g TEMPOL in their food. Plasma samples were analysed for triglycerides, cholesterol, low- and high-density lipoprotein cholesterol, inflammatory cytokines and markers (interleukin-6, IL-6; monocyte-chemotactic protein, MCP-1; myeloperoxidase, MPO; serum amyloid A, SAA; adiponectin; leptin). Plaques in the aortic sinus were analysed for area, and content of collagen, lipid, macrophages and smooth muscle cells.

Results: High fat feeding resulted in marked increases in body mass and plasma lipid levels. Dietary TEMPOL decreased both parameters. In the high-fat-fed mice significant elevations in plasma lipid levels and the inflammatory markers IL-6, MCP-1, MPO, SAA were detected, along with an increase in leptin and a decrease in adiponectin. TEMPOL supplementation reversed these effects. When compared to HFD-fed mice, TEMPOL supplementation increased plaque collagen content, decreased lipid content and increased macrophage numbers.

Conclusions: These data indicate that in a well-established model of obesity-associated hyperlipidaemia and atherosclerosis, TEMPOL had a significant impact on body mass, atherosclerosis, hyperlipidaemia and inflammation. TEMPOL may therefore be of value in suppressing obesity, metabolic disorders and increasing atherosclerotic plaque stability.
1. **Introduction**

Dyslipidaemia plays a major role in many cardiovascular disorders including atherosclerosis [1]. Total blood cholesterol and LDL-cholesterol (LDL-C) are well-established independent risk, and graded factors, for cardiovascular disease and meet the criteria for causality (reviewed [1]). Multiple mechanisms have been proposed to account for the elevated risk associated with increased triglyceride and cholesterol levels, including smooth muscle and endothelial damage, increased lipid accumulation in the arterial wall, impaired vascular repair, and abnormal plasma lipoprotein content and transport [1]. Therapeutic reduction of high-risk lipid fractions are strongly associated with improved outcomes, however, prevention and treatment of obesity (by behavioural modification, pharmaceuticals or surgical procedures), remain critical to reducing the onset of dyslipidaemia and resultant morbidities [1]. Pharmaceuticals also provide tools to investigate pathophysiological mechanisms, and one example is the stable nitroxide free radical TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-N-oxyl radical) which has been shown to modulate sensitivity to radiation, tumourigenesis and diet-induced body mass gain [2].

Nitroxides are stable free radicals with multiple biological effects, including acting as protective agents against oxidation in animal models of inflammation (reviewed [3, 4]). Whilst the mechanisms involved are not fully understood, it has been shown that nitroxides can act: (a) as superoxide dismutase mimetics [5]; (b) as radical scavengers [6-8]); (c) as reducing agents for oxidised heme proteins [3, 4, 9-11]; (d) by inhibiting oxidant formation by the heme enzyme myeloperoxidase (MPO) [10-12]; and (e) by altering the gut microbiome and the accumulation of intestinal tauro-β-muricholic acid which regulates bile acid, lipid and glucose metabolism [13].

TEMPOL (at 10 mg/g of food) has profound inhibitory effects upon body mass gain in C3H mice and prevents obesity-induced changes in leptin levels [2]. A similar effect on mass
gain has recently been reported for mice fed a high fat diet (HFD) [13]. TEMPOL has also been reported to reduce formation of reactive oxygen species induced by diabetes [14], and hypertension [15], with the latter effect ascribed to increased nitric oxide (NO') production by endothelial cells [15] and consequent vasodilation [16]. TEMPOL also protects the endothelium in both spontaneously hypertensive rats and small arteries from visceral fat of obese subjects [16]. Nitroxides have also been postulated to modulate atherosclerosis and vascular inflammation by reducing vascular adhesion molecule-1 (VCAM-1) levels and by decreasing the levels and assembly of specific NADPH oxidase-2 (NOX2) subunits [17].

In the light of the above data we hypothesised that TEMPOL would modulate hyperlipidaemia, inflammation and vascular lipid accumulation in a well-established mouse model of atherogenesis: apoE−/− mice fed a high cholesterol/high fat diet.

2. Methods
2.1 Animals

Animal husbandry of apoE−/− mice (n = 38) was carried out at the National Cancer Institute (Bethesda, MD, USA) using female mice purchased from Taconic Farms (Germantown, NY, USA). At 9 weeks of age two groups were placed on a bacon-flavoured standard diet (SD, AIN-76A) with or without TEMPOL (10 mg/g food; Mitos Pharmaceuticals, Newport Beach, CA, USA) supplemented into the food. Food consumption by the different groups was similar, and qualitative observations indicated greater activity in the TEMPOL-fed groups in line with previous data [2]. The remaining two groups were placed on a bacon-flavoured high-fat diet (HFD; 60% fat calories, F1850) with and without TEMPOL (10 mg/g food) in the food. Both animal chows were purchased from Bioserv (Frenchtown, NJ, USA). All experiments were carried out under the aegis of a protocol approved by the National Cancer Institute Animal Care and Use Committee and were in
compliance with the Guide for the Care and Use Of Laboratory Animal Resource (2011, National Research Council).

2.2 Sample collection and storage

Samples were obtained and stored at -80°C until analysed at the Heart Research Institute (Sydney, Australia) by a blinded observer, for plasma lipids, cytokines, and inflammatory markers. Atherosclerotic lesions in the aortic sinus were processed and analysed as previously described [18, 19].

2.3 Determination of plasma lipids

Total cholesterol and triglycerides were quantified using commercial kits (Wako Diagnostics, Osaka, Japan) in plasma diluted 1:5 with Tris-buffered saline. High- and low-density lipoprotein cholesterol levels (HDL-C and LDL-C respectively) were determined as described previously [20, 21].

2.4 Determination of plasma chemokines, adipokines and inflammatory markers

Mouse Quantikine ELISA kits for tumour necrosis factor-alpha (TNF-α), monocyte chemotactic protein-1 (MCP-1), leptin and resistin levels were purchased from R & D systems (Minneapolis, MN, USA). Other ELISA kits were obtained from the following sources: MPO (Hycult Biotech, Inc., Uden, Netherlands); IL-6 (Ray Biotech, Inc., Norcross, GA, USA); adiponectin (AssayPro, St. Charles, MO, USA); serum amyloid A (SAA; Tridelta Development Ltd., County Kildare, Ireland). Mouse plasma was diluted with the supplied diluents as follows: TNF-α (no dilution), MCP-1 (1:2), leptin (1:10), resistin (1:30), MPO (1:4), IL-6 (1:6), Adiponectin (1:400), SAA (1:200), with assays carried out according to the manufacturer’s guidelines. Data are reported as pg/mL unless indicated otherwise.
2.5 Preparation and analysis of aortic sinus tissue

Three sets of triplicate cross sections were stained with haematoxylin-eosin and photographed as previously [18, 19]. Macrophages (F4/80), smooth muscle cells (alpha-actin) and collagen content were quantified by previously published methods at the Heart Research Institute (Sydney, Australia) [18, 19]. Lipid levels were assessed as white areas (arising from sample delipidation) in images stained with picrosirius red, and were quantified relative to plaque area, as described previously [18, 19].

2.7 Statistical analysis

Statistical analyses were undertaken using Prism 5.03 (GraphPad software, San Diego, CA, USA) by two-way ANOVA analyses followed by Bonferroni’s *post-hoc* tests, with differences considered statistically significant at p < 0.05, unless otherwise stated.

3. Results

3.1 Effect of TEMPOL on body mass

The body mass of the HFD-fed mice increased at a greater rate over the study period than the chow-fed mice (Fig. 1). Inclusion of TEMPOL in the diet significantly reduced this gain in mass for the HFD mice to levels similar to those of the chow-fed mice. TEMPOL also exhibited a significant effect on the body mass gain of the standard chow-fed mice (Fig. 1); however, the extent of the effect was much less than that observed for the HFD.

3.2 Plasma lipids

Plasma triglyceride, total cholesterol, HDL-C and LDL-C levels were significantly elevated in plasma from the HFD- compared to the chow-fed mice (Fig. 1, panels C-F).
TEMPOL attenuated this elevation such that the levels detected in the HFD and TEMPOL supplemented mice were similar to those of the non-HFD-fed apoE\(^{-/-}\) mice. LDL-C levels in the chow-fed mice with TEMPOL were elevated over the corresponding animals without TEMPOL. The total cholesterol levels in these animals were not however elevated.

3.3 Plasma cytokines

MCP-1 levels were not significantly different between the chow- and HFD-fed mice, but the MCP-1 levels detected in the HFD-fed mice supplemented with TEMPOL were significantly lower than those detected in the HFD-fed mice without TEMPOL (Fig. 2). No significant changes were detected in MCP-1 levels between the chow-fed mice with or without TEMPOL. IL-6 levels were significantly elevated in the HFD-fed, compared to the chow-fed, animals, and this increase was markedly attenuated by TEMPOL (Fig. 2). A similar pattern of changes was detected for the inflammatory markers SAA and MPO, with TEMPOL-supplementation significantly blunting the increases detected in the HFD-fed, compared to chow-fed animals.

3.4 Adipokine profile

HFD mice had significantly lower adiponectin levels when compared to chow-fed controls (Fig. 3). TEMPOL returned these levels to those detected in the chow-fed and chow-fed plus TEMPOL animals. Leptin levels showed an inverse pattern, with a significant elevation detected in the HFD-fed, compared to chow-fed mice. TEMPOL significantly reduced this increase (Fig. 3). The adiponectin and leptin values detected in the HFD-fed mice supplemented with TEMPOL were not significantly different to those determined for the chow-fed mice. No significant differences in resistin levels were detected between any of the groups.
3.5 Analysis of atherosclerotic plaques

No statistical differences in atherosclerotic plaque area, compared to total area, were detected between the chow and HFD-fed mice, though a significant decrease in area was detected between the HFD-fed mice supplemented with TEMPOL compared to the chow-fed animals supplemented with TEMPOL (Fig. 4). The collagen content of the lesions from the HFD-fed mice was significantly lower than that detected in the chow-fed animals, and the presence of TEMPOL in the HFD returned these levels to those detected in the chow-fed animals without or with TEMPOL supplementation (Fig. 4). There were no differences detected in plaque area occupied by α-actin-positive cells though there was a clear trend to higher levels in the HFD-fed mice with TEMPOL supplementation compared to its absence (Fig. 4). TEMPOL supplementation significantly increased the levels of tissue macrophages, as quantified by F4/80 staining (Fig. 5) and decreased the levels of lipid detected in the plaques from the HFD-fed mice (Fig. 5).

4. Discussion

Previous studies have indicated that TEMPOL can act as a protective agent against oxidative damage in multiple pathologies (reviewed [3, 4]) and does not to affect cell growth or induce toxicity at ≤ 1 mM in cell media [22] or at 10 mg/g food in animal studies (~ 58 mM) [2, 23]. In the light of this data we investigated whether this compound had therapeutic potential against the deleterious effects of a high-fat diet (HFD) in apoE<sup>−/−</sup> mice, a well-established model of chronic inflammation and atherosclerosis.

As expected, feeding with the HFD was accompanied by elevated plasma triglycerides, total cholesterol, LDL-C, HDL-C, IL-6, SAA, MPO and leptin, decreased levels of adiponectin, and significant atherosclerosis in the aortic sinus. Inclusion of TEMPOL in the
animal chow had profound, and in many cases novel, effects on the majority of these parameters, indicating that this compound has marked antioxidant, anti-inflammatory and anti-hyperlipidaemic effects.

As observed in previous studies with other mouse strains fed either normal chow [2, 23] or a HFD [13], TEMPOl had a marked inhibitory effect on the gain in body mass associated with HFD feeding. In these previous studies it has been established that TEMPOl does not affect food consumption but is associated with increased physical exercise on a running wheel [2, 23].

Of particular interest and novelty are the observations that TEMPOl decreases the elevated plasma levels of triglycerides, total cholesterol, LDL- and HDL-cholesterol, leptin, MCP-1, IL-6, SAA and MPO, and increases the adiponectin levels detected in the apoE-/- mice, back to the levels detected in the chow-fed animals. No effect was detected on plasma resistin levels. With regard to the atherosclerotic lesions quantified in the aortic sinus, TEMPOl did not significantly change the plaque area, but did enhance the collagen and α-actin content, as well as the area occupied by macrophage cells (as quantified by F4/80 staining), and decreased the lipid content. These changes in plaque composition have been reported to be associated with a more stable lesion phenotype [24].

The current data suggest that TEMPOl may induce these changes via multiple mechanisms. It has been show that elevated inflammatory markers are present in obesity-related metabolic complications both in animals and in humans. Higher plasma levels and protein expression of TNF-α has been reported in obese humans [25] and animals [26, 27], and increased TNF-α levels and iNOS expression are associated with endothelial dysfunction in small arteries present in the visceral fat of obese patients [28] these changes, together with vascular superoxide radical generation, have been reported to be attenuated by TEMPOl [28]. TEMPOl has well documented superoxide dismutase mimetic activity [3-5] which account
for the lower levels of vascular superoxide, but it has also been reported that TEMPOL modulates the mRNA and protein levels of the p47phox (but not other) subunits of vascular NADPH oxidase (NOx2) which generates superoxide, with this resulting in decreased inflammation, and a reduced accumulation of neutrophils and MPO [29].

Neutrophils are present in early atherosclerotic lesions [30, 31] and are known to be a major source, together with monocytes, of the MPO present at sites of inflammation, though there is also some evidence for MPO release from tissue macrophages [32, 33]. MPO is a major source of reactive oxidants within atherosclerotic lesions [32] and there is considerable evidence to support a major role for this enzyme and the oxidants that it generates in the development of cardiovascular disease [34, 35]. TEMPOL and related nitroxides have been shown to directly inhibit oxidant formation by MPO via interactions with the enzymatic cycle of the enzyme [10, 11, 36], and this activity is believed to be responsible, at least in part, for the inhibition of MPO-mediated inflammation and tissue damage.

The lesion data obtained for the aortic sinus do not correlate directly with the observed obesity and dyslipidaemia, with TEMPOL having no effect on lesion area. This is in contrast to a recent study [17] where a significant diminution of lesion area was detected in apoE−/− mice fed a fructose-rich diet for 8 weeks with TEMPOL present in drinking water for the final 4 weeks. The lack of effect in the current study may reflect differences between these models. In particular, the fructose-fed mice did not have elevated total cholesterol (unlike most human atherosclerosis and the current study), though triglycerides were elevated [17], furthermore this previous study used en face analysis of lesions, rather than plaque size in the aortic sinus, as used here.

The mechanism by which TEMPOL induces changes in plaque composition in the HFD mice compared to those without TEMPOL is less clear, with the data indicating significantly increased plaque collagen and macrophage levels, a trend towards higher
numbers of α-actin positive cells, and a decreased plaque lipid content. This decrease in plaque extracellular lipid content may reflect the lower levels of plasma lipids, less advanced foam cell formation, and higher lesion macrophage numbers as a result of a decreased extent of apoptosis and necrosis of lesion macrophages [37, 38]. The reduction in lipid content and the higher collagen (a marker of extracellular matrix content) and α-actin induced by TEMPOL, appear to reflect a more stable and less advanced plaque phenotype [24]. Similar changes, with lower lipid levels and foam cell numbers, and reduced inflammation, apoptosis and necrotic core formation, have been observed in the aortic sinus of apoE−/− mice fed a pro-atherogenic diet and treated with D-carnosine octylester [39], and in (streptozotocin-induced) diabetic apoE−/− supplemented with carnosine [18]. In the latter case decreased plasma triglycerides were also detected. A TEMPOL-induced reduction in diet-induced dyslipidaemia has been reported previously for lean (LZR) and obese Zucker rats (OZR) fed a HFD [40], and in Fischer 344 rats [41].

In addition to direct effects on cells and processes with the vascular wall TEMPOL may also affect adipocytes and the gut microbiome. Thus SAA is expressed at high concentrations by adipocytes from obese subjects [42], and plasma SAA levels are increased in atherosclerosis, diabetes and obesity [43-45]. This is consistent with the current observations, where the HFD-fed mice had markedly elevated SAA and leptin, and lower adiponectin levels, that were corrected by TEMPOL. Adiponectin exerts strong anti-inflammatory and athero-protective effects on vascular tissue, and has an insulin-sensitising effect on tissues associated with glucose and lipid metabolism [46, 47]. Lower adiponectin levels have also been reported in humans with obesity, diabetes and coronary artery disease [48], and hyperleptinaemia is common in obese people, and indicative of greater adipose tissue reservoirs and excess secretion causing leptin resistance [49]. These data supports the hypothesis that TEMPOL targets adipose tissue [50]. TEMPOL has also been shown to
modulate the gut microbiome [13] with the accumulation of intestinal tauro-β-muricholic acid providing a potential rationale for the effects of TEMPO, as the former is a farnesoid X receptor (FXR) nuclear receptor antagonist involved in the regulation of bile acid, lipid and glucose metabolism.

Overall these data demonstrates that TEMPO, a well-tolerated nitroxide, can modulate the gain in body mass, dyslipidaemia and inflammation detected in apoE<sup>-/-</sup> mice fed a HFD, with this being associated with changes in atherosclerotic plaque composition commensurate with a more stable phenotype.

**Conflict of interest**

The authors have declared no conflict of interest.

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Figure Legends

**Fig. 1.** Body mass and plasma lipid parameters for apoE<sup>−/−</sup> mice that were fed either a chow or HFD, with or without TEMPOL supplementation. (A) Mean body mass (g) of mice over the time course of the study: □, apoE<sup>−/−</sup> mice fed HFD; ■, apoE<sup>−/−</sup> mice fed HFD plus added TEMPOL; ○, apoE<sup>−/−</sup> mice fed standard chow; ●, apoE<sup>−/−</sup> mice fed standard chow plus added TEMPOL. Data were compared using the non-parametric Mann-Whitney U-test of significance using WinSTAT® for Excel, with comparisons made between HFD vs. HFD plus TEMPOL (* p < 0.001) and standard chow vs. standard chow plus TEMPOL († p < 0.001). Error bars (± SD) are only shown for the mice fed HFD, and HFD plus added TEMPOL; in some cases these are smaller than the symbols. Panel (B), plasma triglycerides; (C) total cholesterol; (D) HDL-cholesterol; (E) LDL-cholesterol. Data in (C)-(E) are given as mmol/L. For panels (B) – (E) values are expressed as mean ± SEM and * indicates statistical difference against the chow-fed group, + versus the chow-fed group administered TEMPOL, and # versus the HFD-fed mice using two-way ANOVA analysis followed by Bonferroni’s post-hoc test.

**Fig. 2.** Plasma cytokines and inflammatory agents in apoE<sup>−/−</sup> mice that were fed either a chow or HFD, with or without TEMPOL supplementation. Plasma cytokines; MCP-1 (Panel A) and IL-6 (B) and inflammatory agents; SAA (C) and MPO (D) were measured in mice that were fed normal chow or a HFD, with or without TEMPOL supplementation. Values are expressed as mean ± SEM. * Indicates statistical difference against the chow-fed group, + versus the chow-fed group administered TEMPOL, and # versus the HFD-fed mice using two-way ANOVA analysis followed by Bonferroni’s post-hoc test.
Fig. 3. Plasma levels of (A) adiponectin, (B) leptin, and (C) resistin, in apoE−/− mice fed either a chow or HFD, with or without TEMPOL supplementation. Values are expressed as mean ± SEM. * Indicates statistical difference against the chow-fed group, and # versus the HFD-fed mice using two-way ANOVA analysis followed by Bonferroni’s post-hoc test.

Fig. 4. (A) Plaque area, (B,C) collagen content, and (D) content of α-actin positive cells (each expressed as a percentage over the total area) in the aortic sinus in apoE−/− mice that were fed either a chow or HFD, with or without TEMPOL supplementation. Representative images of atherosclerotic plaques stained for collagen with picrosirius red in sinus cross-sections are shown in panel (C) and quantified in panel (B) from animals fed control chow (CTL), control chow plus TEMPOL (CTL + T), high-fat diet (HFD) or HDF plus TEMOPL (HFD + T). Values are expressed as mean ± SEM. * Indicates statistical difference against the chow-fed group, + versus the chow-fed group administered TEMPOL, and # versus the HFD-fed mice using two-way ANOVA analysis followed by Bonferroni’s post-hoc test.

Fig. 5. (A,C) Plaque macrophage (F4/80 staining) and (B) lipid content in the aortic sinus in apoE−/− mice that were fed either a chow or HFD, with or without TEMPOL supplementation. Panel (A) represents representative images of atherosclerotic plaques stained with antibody F4/80 to detect macrophages (pink staining); legend as Fig. 4. Quantification of macrophage staining is expressed relative to plaque area in panel (B). Lipid levels were assessed as white areas (arising from sample delipidation) in images stained with picrosirius red (cf. Fig. 4) and are quantified relative to plaque area in panel (C). Values are expressed as mean ± SEM. * Indicates statistical difference against the chow-fed group, + versus the chow-fed group administered TEMPOL, and # versus the HFD-fed mice using two-way ANOVA analysis followed by Bonferroni’s post-hoc test.
References


Figure 1
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