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Inhibition of inflammatory response in transgenic *fat-1* mice on a calorie-restricted diet

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Abstract

Both $n-3$ fatty acids ($n-3$ FA) and calorie-restriction (CR) exert anti-inflammatory effects in animal models of autoimmunity and inflammation. In the present study we investigated the synergistic anti-inflammatory effects of $n-3$ FA and CR on LPS-mediated inflammatory responses using *fat-1* transgenic mice that generate $n-3$ FA endogenously. Wild-type (WT) and *fat-1* mice were maintained on *ad libitum* (AL) or CR (40% less than AL) diet for 5 mo; splenocytes were cultured *in vitro* with/without LPS. Our results show: (i) no difference in body weights between WT and *fat-1* mice on AL or CR diets, (ii) lower $n-6/n-3$ FA ratio in splenocytes from *fat-1* mice on both AL and CR diets, (iii) significant reduction in NF-κB (p65/p50) and AP-1 (c-Fos/c-Jun) DNA-binding activities in splenocytes from *fat-1*/CR mice following LPS treatment, and (iv) significant reduction in κB- and AP-1-responsive IL-6 and TNF-α secretion following LPS treatment in splenocytes from *fat-1*/CR mice. The inhibition of LPS-mediated effects was more pronounced in *fat-1*/CR mice when compared to *fat-1*/AL or WT/CR mice. These data show that transgenic expression of *fat-1* results in decreased pro-inflammatory $n-6$ FA, and demonstrate for the first time that splenocytes from *fat-1* mice on CR diet exhibit reduced pro-inflammatory response when challenged with LPS. These results suggest that $n-3$ lipids with moderate CR may confer protection in autoimmune and inflammatory diseases.

Keywords: *Fat-1*; $n-3$ fatty acids; Calorie-restriction; Cytokines; NF-κB; AP-1; Inflammation; Splenocytes

Inflammation is the body's immediate response to initiate a repair mechanism when subjected to a foreign challenge (e.g., pathogens and toxins) or tissue injury. A normal inflammatory response is self-limiting and involves down-regulation of pro-inflammatory mediators, and an increase in anti-inflammatory mediators. However, overt inflammatory response contributes to the pathogenesis of several inflammatory diseases that include rheumatoid arthritis (RA) and inflammatory bowel disease (IBD) [1,2].

Inflammatory stimuli such as bacterial lipopolysaccharide (LPS) and interferon-γ produce a variety of pro-inflammatory cytokines; tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, and other inflammatory mediators like prostaglandin (PG)E_2 and nitric oxide (NO) that are involved in the pathogenesis of many inflammation-associated human diseases [3]. Anti-inflammatory agents such as non-steroidal drugs reduce the inflammatory response by suppressing the production of these inflammatory mediators and attenuate progression of autoimmune and inflammatory disorders [3].

Nuclear factor-kappa B (NF-κB) is a ubiquitous multisubunit transcription factor and is involved in the induction of several pro-inflammatory cytokines and enzymes that are critically involved in the pathogenesis of chronic inflammatory diseases [4]. NF-κB is activated as a consequence of phosphorylation, ubiquitination, and proteolytic

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degradation of the inhibitory subunit IκB. The liberated NF-κB translocates into nuclei and binds to κB motifs in the promoters of pro-inflammatory genes such as TNF-α, IL-1β, and IL-6 leading to their induction [5]. Since anti-inflammatory drugs suppress the expression of these pro-inflammatory genes by inhibiting the NF-κB activation pathway, inhibition of NF-κB could be a potential target in regulation of inflammation-associated human diseases.

Dietary intervention with long-chain polyunsaturated fatty acids has a profound effect on physiological processes and inflammatory diseases [6]. At present, *n*−6 fatty acids (*n*−6 FA) form a major part of the fatty acid intake in Western-style diets [7]. Intake of *n*−3 fatty acids (*n*−3 FA) like eicosapentaenoic acid (EPA; 20:5*n*−3) and docosahexaenoic acid (DHA; 22:6*n*−3), however, remains comparatively low. This results in significant increase in *n*−6/*n*−3 FA ratio in the diet [7] which may promote the pathogenesis of many age-related diseases including CVD and cancer [7,8]. Interestingly, *n*−3 FA or low *n*−6/*n*−3 FA ratio has been shown to attenuate several inflammatory disorders in rodent models [8–10]. *n*−3 FA inhibit activity and expression of pro-inflammatory mediators [6,7,11]. Inhibition of NF-κB activation has been proposed as one of the key mechanisms involved in decreased inflammatory response with *n*−3 FA [9,12]. The AP-1 family of transcription factors which are involved in the up-regulation of many immune and inflammatory genes [13] has also been shown to be down-regulated with *n*−3 FA [14,15]. Some clinical trials also support the findings of animal studies and report beneficial effects of *n*−3 FA in reducing clinical symptoms of inflammatory diseases like RA [16] and IBD [17].

Caloric restriction (CR) refers to the reduction of caloric intake without reducing essential nutrients in the diet. CR (diet ~30–40% less than *ad libitum* intake) significantly extends life span in rodents and other species [18]. Apart from slowing the aging process, CR provides many health benefits by decreasing or delaying many of the age-related diseases [19]. There is evidence to suggest that inhibition of inflammatory response and age-related inflammation by CR are key mechanisms involved in these beneficial effects [20–22]. CR modulates the activities and expressions of several pro-inflammatory cytokines like TNF-α, IL-1β, IL-6, and synthesis of other pro-inflammatory mediators [20,21,23]. It has been suggested that modulation of NF-κB by CR could be involved in inhibition of inflammatory response through these mediators [20,23].

Transgenic *fat-1* mice carry the *fat-1* gene from *Caenorhabditis elegans*, which encodes for an *n*−3 desaturase enzyme that can synthesize *n*−3 FA endogenously using *n*−6 FA as substrate [24] leading to a significant decrease in tissue *n*−6/*n*−3 FA ratio, compared to wild-type (WT) mice. Thus, *fat-1* mouse provides a unique model to study the effect of *n*−6/*n*−3 FA ratio on inflammatory processes and molecular mechanisms without providing *n*−3 FA exogenously. A recent study showed significant protection against dextran sodium sulfate-induced colitis model of IBD in *fat-1* mice [25].

We previously showed that exogenously provided *n*−3 FA + CR significantly extends life span of short-lived autoimmune-disease prone NZB/W mice through inhibition of age-related increase in inflammation and decline in antioxidant status [11,22]. However, the combined effect of *n*−3 FA and CR on inhibition of inflammatory response following an endotoxin challenge, particularly in normal strain of mice, is not known and remains to be investigated. Hence, the present study was designed to investigate the *in vitro* inhibition of LPS-stimulated inflammatory response in spleen cells of *fat-1* transgenic mice on CR diet.

Materials and methods

Animals and experimental diets. Heterozygous *fat-1* mice on C57BL/6 background [24] were previously described. These animals show no phenotypic changes. *Fat-1* heterozygous and the non-transgenic litter mate controls were used in the study. Presence of *fat-1* gene was confirmed by genotyping and fatty acid composition of tails by gas chromatography (GC). Age- and weight-matched mice were housed in a laboratory animal care facility in cages (3–4 mice/cage) and fed semi-purified AIN-93 M diets containing 10% corn oil (CO) (MP Biomedicals, Irvine, CA). CO is high in linoleic acid (18:2*n*−6) and fat-1 mice convert *n*−6 FA to *n*−3 FA. The composition of the semi-purified diet has been previously reported [26]. At 6 months of age, weight-matched mice were divided into *ad libitum* (AL) and calorie-restricted (CR, food provided gradually lowered to 40% less than AL) groups resulting in 4 dietary groups — WT/AL, *fat-1*/AL, WT/CR and *fat-1*/CR. Mice ($n = 7$–8/group) were maintained on the experimental diets for 5 mo until sacrifice. All studies were approved by the Institutional Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

Isolation of splenocytes and culture. Spleens were aseptically removed and placed in 5 ml RPMI 1640 media (Gibco, Grand Island, NY) supplemented with 25 mmol Hepes/L, 2 mmol glutamine/L, 10,0000 U penicillin/L, and 100 mg streptomycin/L (Gibco, Grand Island, NY). Single cell suspensions were made and cultures were set up with/without bacterial lipopolysaccharide (LPS, 5 μg/ml). After 24 h, cells and culture medium were collected together and centrifuged at 2000 rpm for 5 min. The cell pellets and culture supernatants were stored at −80 °C [9].

Cytokine measurement in cultured splenocyte supernatants. TNF-α and IL-6 were measured by ELISA using BD OptEIA™ ELISA kits from BD Biosciences Pharmingen (San Diego, CA) as previously described [26].

NF-κB and AP-1 transcription factor activity assay. Extraction of nuclear proteins and transcription factor activation and subunit compositions were determined as previously described using a commercially available ELISA-based assay [27].

Analysis for fatty acids in splenocytes. Splenocytes (~1×10^6 cells) were used for the extraction of fatty acids as described previously [26]. Fatty acid methyl esters were identified by comparison of retention times with fatty acid methyl ester standard (FIM-FAME-7; Matreya, Inc., Pleasant Gap, PA). Results are expressed as ratio of *n*−6 to *n*−3 FA and ratio of AA to (EPA + DHA + docosapentaenoic acid (DPA)) as previously described [24].

Statistical analysis. Values are expressed as means ± SEM of 7–8 mice/group. Analysis of variance (ANOVA) was used to assess the statistical significance of the differences of the means, with significance established at $p < 0.05$.

Results

Body weight

Body weights increased gradually in WT/AL and *fat-1*/AL mice but there was no difference between the 2 groups