

# The *fat-1* transgene in mice increases antioxidant potential, reduces pro-inflammatory cytokine levels, and enhances PPAR $\gamma$ and SIRT-1 expression on a calorie restricted diet

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**Key words:** lipids, n-3 fatty acids, calorie restriction, inflammation, oxidative stress, aging, TNF $\alpha$ , IL-6, IL-1 $\beta$ , PPAR $\gamma$ , SIRT-1

**Abbreviations:** FA, fatty acids; PUFA, poly unsaturated fatty acids; CR, calorie-restriction; WT, wild-type; AL, ad libitum; CAT, catalase; SOD, superoxide dismutase; GPX, glutathione peroxidase; ROS, reactive oxygen species; AA, arachidonic acid; EPA, eicosapentanoic acid; DHA, docosahexanoic acid; HD, heart disease; CRP, c reactive protein; AMs, adhesion molecules; FO, fish oil; BCA, bicinchoninic acid; DPA, docosapentaenoic acid

Both n-3 fatty acids (FA) and calorie-restriction (CR) are known to exert anti-inflammatory and anti-oxidative effects in animals and humans. In this study, we investigated the synergistic anti-inflammatory and anti-oxidative capacity of n-3 FA and CR using *Fat-1* transgenic mice (*Fat-1*) that are capable of converting n-6 FA to n-3 FA endogenously. Wild type (WT) and *Fat-1* mice were maintained on ad libitum (AL) or CR (40% less than AL) AIN-93 diet supplemented with 10% corn oil (rich in n-6 FA) for 5 months. Significantly lower levels of n-6/n-3 FA ratio were observed in serum, muscle and liver of *Fat-1* mice fed AL or CR as compared to that of WT mice fed AL or CR. Muscle catalase (CAT), super oxide dismutase (SOD), glutathione peroxidase (GPX) activities, and liver CAT and SOD activities were found higher in *Fat-1* mice as compared to that of WT mice. These activities were more pronounced in *Fat-1*/CR group as compared to other groups. Serum pro-inflammatory markers, such as tumor necrosis factor (TNF) $\alpha$ , interleukin (IL)-1 $\beta$  and IL-6 were found lower in *Fat-1* mice, as compared to that of WT mice. This anti-inflammatory effect was also more pronounced in *Fat-1*/CR group as compared to that of other groups. Furthermore, significantly higher levels of peroxisome proliferator-activated receptor (PPAR) $\gamma$  and life prolonging gene, sirtuin (SIRT)-1 expression were found in liver of *Fat-1*/CR mice, as compared to that of WT/CR mice. These data suggest that n-3 FA along with moderate CR may prolong lifespan by attenuating inflammation and oxidative stress.

## Introduction

Oxidative stress is caused by an imbalance between the productions of reactive oxygen species (ROS) and ROS neutralizing anti-oxidant enzymes. All forms of life maintain a reducing environment within their cells. This reducing environment is preserved by enzymes that maintain the reduced state through a constant input of metabolic energy. Disturbances in this normal redox state can cause toxic effects through the production of peroxides and free radicals that damage all components of the cell, including proteins, lipids and DNA. 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 downregulation of pro-inflammatory mediators,

and an increase in anti-inflammatory mediators. However, an over inflammatory response contributes to the pathogenesis of several inflammatory diseases.<sup>1</sup> Inflammation and oxidative stress are associated with aging, as well as a vast majority of diseases, including cancer, cardiovascular diseases, hypertension, atherosclerosis, diabetes, osteoporosis, arthritis, sarcopenia, parkinson's disease, Alzheimer's disease and chronic fatigue syndrome. Therefore, strategies that can modulate both inflammation and oxidative stress will ensure a reduction of pathological conditions thereby promoting healthy aging and extension of life span.

n-3 fatty acids (FA), along with n-6 FA, are essential polyunsaturated FA for optimal health. The western diet is abundant in n-6 FA, mainly from vegetable oils rich in linoleic acid and arachidonic acid (AA). The n-6 FA, especially AA, is a precursor

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of prostaglandins, leukotrienes and related compounds that influence the synthesis of eicosanoids, which may enhance inflammation.<sup>2</sup> Anti-oxidative and anti-inflammatory properties of n-3 FA are well established. The consumption of fish, rich in n-3 FA, is recommended for its health benefits to protect against heart disease, diabetes and potentially cancer.<sup>3</sup> Consuming increased amounts of long chain n-3 FA results in a partial replacement of the AA in cell membranes by eicosapentanoic acid (EPA 20:5) and docosahexanoic acid (DHA 22:6).<sup>4</sup> This leads to a decreased production of AA-derived mediators. We and others earlier reported the inhibition of pro-inflammatory cytokines production by n-3 FA in cells and tissues.<sup>2,5-7</sup> NFκB is the key regulator of inflammatory pathways. We and others have reported that n-3 FA suppress the activation of NFκB in cells and tissues.<sup>2,6</sup> It has been also reported that n-3 FA inhibit oxidative stress.<sup>8</sup> It is widely accepted that fish oil (FO), rich in n-3 poly unsaturated fatty acids (PUFA), protect against several types of cardiovascular diseases, such as myocardial infarction, arrhythmia, atherosclerosis and hypertension.<sup>9</sup> A recent health study in nurses revealed that a higher consumption of n-3 FA was associated with a lower risk of heart disease (HD), and particularly HD related deaths.<sup>10</sup> Furthermore, the blood level of n-3 FA is considered as a predictive biomarker for sudden death in men.<sup>11</sup> n-3 FA has also been reported to decrease plasma triglycerides, and increase serum HDL-cholesterol,<sup>12</sup> which is associated with more efficient reverse cholesterol transport and a reduced risk of coronary heart disease.<sup>13</sup> Our own studies, either with CR or n-3 FA, or recently with their combination, have shown several beneficial effects on antioxidant enzyme levels in BW and MRL/lpr mice.<sup>14</sup>

CR is considered as the only established anti-aging experimental paradigm. CR's anti-aging effects are thought to be due mainly to its powerful anti-inflammatory, resistance against oxidative stress and its ability to maintain a proper cellular redox status as evidenced by suppressed oxidative damage to lipids, DNA and proteins.<sup>15</sup> The antioxidant defense system decreases with age and CR is shown to prevent these decreases.<sup>16</sup> Thus, the effects of short- and long-term CR are believed to be due to its powerful anti-oxidative action.<sup>15</sup> Recent evidence has also documented CR's anti-inflammatory action, as shown in its modulation of pro-inflammatory genes such as TNFα, IL-1, IL-6, c reactive protein (CRP), adhesion molecules (AMs), iNOS and COX-2 through the NFκB signaling pathway.<sup>15</sup> Furthermore, it has been demonstrated the capacity of long-term 40% CR to attenuate the increase in circulating levels of CRP and reduced IL-6 secretion from adipose tissue.<sup>15</sup> Animal studies have shown repeatedly that CR enhances metabolic efficiency, decreases inflammation and increases stress resistances over the lifespan, thereby delaying the onset of age-related pathological conditions.

The lifespan extension in yeast has been shown to be associated with increased activity of the Nicotinamide adenine dinucleotide (NAD)<sup>+</sup>-dependent protein deacetylase Sir2.<sup>17,18</sup> Mammals express at least seven Sir2 orthologs, designated sirtuins (Sirt1-7), varying in cellular expression and function.<sup>19</sup> Mammalian sirtuins have been shown to deacetylate and regulate a large array of substrates including p53, FOXO, tubulin and metabolic proteins such as PGC-1α, PPARγ and acetyl-CoA synthetase.<sup>20</sup> Thus,

sirtuins have a significant potential to impact metabolic pathways involving glucose homeostasis, the insulin/IGF-1 signaling pathway, and stress response pathways associated with DNA damage and oxidative stress.<sup>21</sup> Pointing to a possible role of sirtuins in mammalian aging and metabolism, two independent studies have demonstrated recently that oral administration of resveratrol (a natural compound enriched in grapes and identified as a direct activator of sirtuins) can reverse the pathophysiologic effects of high fat diet and restore life span.<sup>22,23</sup> The PPARγ is a regulator of anti-inflammatory genes.<sup>24</sup> Very recently, upregulation of PPARγ has been shown to be associated with reduction of oxidative stress.<sup>24-27</sup>

In 2004, Kang et al. generated transgenic Fat-1 mouse (Fat-1) on C57BL6 background carrying the *fat-1* gene from *Caenorhabditis elegans*, which encodes for an n-3 desaturase enzyme that can synthesize n-3 FA from n-6 FA.<sup>15,28</sup> Different tissues of Fat-1 mice show increase in n-3 FA and decrease in n-6 FA leading to a significant decrease in n-6/n-3 FA ratio. Thus, Fat-1 transgenic mice have an n-6/n-3 FA ratio of ~1:1 compared to wild-type mice with ratio of 20-30:1. Thus, the Fat-1 mouse represents a significant advance in the development of a more sophisticated research model to investigate the effect of n-3 FA and n-6/n-3 FA ratio on physiological parameters and molecular mechanisms without providing exogenous n-3 FA in the form of fish oil (FO). Recent studies with Fat-1 mouse have already yielded interesting results including reduction of inflammation, bone loss, lung injury, colitis, colon cancer, melanoma growth, invasiveness of lung cancer cells, and seizure susceptibility in Fat-1 transgenic mice.<sup>2,29-34</sup> We and others have shown that Fat-1 mice attenuate the inflammatory response following a bacterial lipopolysaccharide (LPS) challenge.<sup>29</sup> In this study, we used Fat-1 mice to examine if there is any synergistic effect of endogenously synthesized n-3 FA and 40% CR on the anti-inflammatory and anti-oxidative capacity in mice. Fat-1 mice exhibited a higher n-3 FA and lower n-6/n-3 FA ratio in different tissues. There was a synergistic effect of endogenous n-3 FA and CR on lowering the production of pro-inflammatory cytokines and on enhancing the production of anti-oxidant enzymes, as well as enhancing the expression of PPARγ and the life prolonging gene SIRT-1, thereby may delay the progression of aging-associated pathological consequences.

## Results

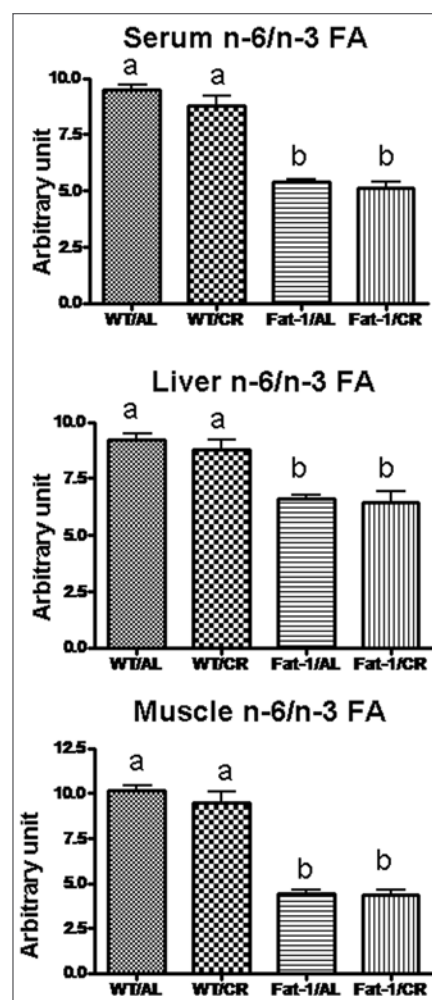
**Effect of *fat-1* transgene and CR on fatty acid composition of serum, muscle and liver.** The *fat-1* gene of *C. elegans* encodes an n-3 fatty-acid desaturase enzyme that converts n-6 FA to n-3 FA and which is absent in most animals, including mammals.<sup>4</sup> Both WT and Fat-1-transgenic littermates born to the same mother were maintained on an identical diet that was high in n-6 FA, but deficient in n-3 FA. However, the fatty-acid profiles of the two groups turned out to be quite different in different tissues analyzed by GC. In this study, we performed GC to determine the effect of *fat-1* transgene on fatty acid composition of serum, muscle and liver. Our results showed a decrease in n-6/n-3 FA ratio ((18:2n-6 + 20:4n-6 + 22:4n-6 + 22:5n-6)/(18:2n-6 + 20:4n-6

+ 22:4n-6 + 22:5n-6)) in Fat-1/AL and Fat-1/CR mice, as compared to WT/AL and WT/CR mice (Fig. 1). However, CR did not make any change in the fatty acid composition of tissues (Fig. 1). During this dietary regime, Fat-1 mice had significantly higher amounts of n-3 FA, such as eicosapentaenoic acid (EPA) (20:5 FA), docosapentaenoic acid (DPA) (22:5 FA) and docosahexaenoic acid (DHA) (22:6 FA), in serum, muscle and liver as compared to that of WT mice (Data not shown). However, the reduction of n-6 FA in Fat-1 mice was not as dramatic as of increase in n-3 FA. There was no difference found in linoleic acid (LA) (18:2 n-6 FA) level in different tissues between the groups (Data not shown). Reduced level of arachidonic acid (AA) (20:4 FA), 22:4 FA and 22:5 FA were found in different tissues of Fat-1 mice as compared to that of WT mice (Data not shown). This indicates that the transgene is functionally active in vivo and is transmittable. The relative ratio between n-6 and n-3 FA is an important determinant in the overall health benefits of consuming n-3 FA.<sup>35</sup> The n-6 to n-3 FA ratio consumed in the present western diet is between 10:1 and 20:1; however, our ancestors had a diet closer to 1:1.<sup>36</sup> A lower n-6 to n-3 FA ratio may be optimal for one's health. Our findings suggest that a decrease in n-6/n-3 FA ratio in Fat-1 mice on AL and CR diets may contribute to the reduction of inflammation and oxidative stress.

**Effect of endogenous n-3 FA and CR on serum pro-inflammatory cytokines levels.** Both n-3 FA and CR are known to be anti-inflammatory. We determined the serum TNF $\alpha$ , IL-6 and IL-1 $\beta$  using standard ELISA techniques. We found both endogenous n-3 FA and CR had the capacity to reduce the serum TNF $\alpha$ , IL-6 and IL-1 $\beta$  levels (Fig. 2). However, enhanced reduction of serum TNF $\alpha$ , IL-6 and IL-1 $\beta$  was observed in Fat-1/CR group, as compared to that of WT/CR group, WT/AL and Fat-1/AL groups (Fig. 2). These results suggest that endogenous n-3 FA and CR may have a synergistic effect on lowering the production of pro-inflammatory cytokines in animals. Further, reduced levels of pro-inflammatory cytokines levels indicate that Fat-1 mice on CR diet may exert less inflammatory response, which may improve pathological conditions during the progression of aging.

**Effect of endogenous n-3 FA and CR on anti-oxidant enzymes activity in muscle.** Oxidative stress plays a major role in the progression of aging and diseases and the level of oxidative stress depends on the balance between ROS and antioxidant enzymes activity. In this study, we have analyzed endogenous n-3 FA and CR on the CAT, SOD and GPX activity in muscles. n-3 FA and CR both enhanced the activity of CAT, SOD and GPX in muscles (Fig. 3). However, the activity of CAT, SOD and GPX was further augmented in muscles of Fat-1/CR mice, as compared to that of WT/CR, WT/AL and Fat-1/AL mice (Fig. 3). These results indicate that n-3 FA and CR may have a synergistic effect on augmenting the activity of anti-oxidant enzymes, which will neutralize the free radicals generated during the course of life and reduce the oxidative damage to DNA, lipids and proteins, thereby improving the pathological conditions during the progression of aging.

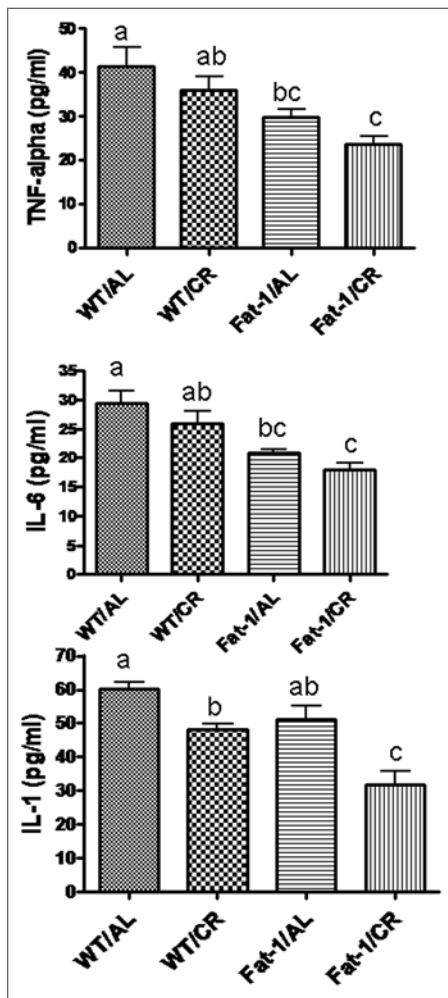
**Effect of endogenous n-3 FA and CR on anti-oxidant enzymes activity in liver.** We have also analyzed the effect of endogenous



**Figure 1.** Polyunsaturated fatty acids (FA) profile of serum, liver and muscle from wild type (WT) and Fat-1 transgenic (Fat-1) mice fed AIN-93M diet supplemented with 10% corn oil either on ad libitum (AL) or calorie restriction (CR) diet. Results are expressed as ratio of n-6 FA: n-3 FA. Ratio of n-6/n-3 FA is expressed as  $(18:2n-6 + 20:4n-6 + 22:4n-6 + 22:5n-6)/(18:3n-3 + 20:5n-3 + 22:5n-3 + 22:6n-3)$ . Each bar represents the mean  $\pm$  SEM (n = 8–10/group). Value with different superscripts are significantly different at  $p < 0.05$  by Newman-Keuls' one way ANOVA with multiple comparison test. WT/AL, wild type ad libitum; WT/CR, wild type calorie restriction; Fat-1/AL, Fat-1 transgenic ad libitum; Fat-1/CR, Fat-1 transgenic calorie restriction.

n-3 FA and CR on CAT and SOD activity in the liver. Similar to that of muscle, a slight increase in the activity of CAT and SOD was seen in both endogenously produced n-3 and CR mice liver (Fig. 4). However, the CAT and SOD activities were augmented in the liver of Fat-1/CR mice, as compared to that of WT/CR mice (Fig. 4). The results indicate a synergistic effect of n-3 FA and CR on augmenting the activity of anti-oxidant enzymes.

**Effect of endogenous n-3 FA and CR on the SIRT-1 expression in liver.** The anti-aging effects of human homologues of sirtuins, SIRT1-7, have been suggested by animal and human association studies.<sup>37</sup> It has been also reported that CR promotes mammalian cell survival by inducing SIRT1.<sup>38</sup> However, the effect of n-3 FA or combined effect of n-3 FA and CR were



**Figure 2.** Endogenous n-3 FA and CR reduce serum tumor necrosis factor (TNF) $\alpha$ , interleukin (IL)-6 and IL-1 $\beta$  levels: After 5 months of dietary intervention, blood was collected and serum was prepared. TNF $\alpha$ , IL-6 and IL-1 $\beta$  levels in serum were analyzed using TNF $\alpha$ , IL-6 and IL-1 $\beta$  ELISA assay kits. Each bar represents the mean  $\pm$  SEM (n = 8-10/group). Value with different superscripts are significantly different at  $p < 0.05$  by Newman-Keuls' one way ANOVA. WT/AL, wild type ad libitum; WT/CR, wild type calorie restriction; Fat-1/AL, Fat-1 transgenic ad libitum; Fat-1/CR, Fat-1 transgenic calorie restriction.

not known. As reported by others, we also found an increase in SIRT-1 expression, due to CR in mice (Fig. 5). However, dramatic increase in SIRT-1 expression was noted in Fat-1/CR mice, as compare to that of WT/CR mice. These results support the notion that CR extends life span. These data also indicate that CR, along with n-3 FA supplementation, may extend the life span even further.

**Effect of endogenous n-3 FA and CR on the PPAR $\gamma$  expression in liver.** PPAR $\gamma$  is the key regulator of glucose, insulin and lipid metabolism. PPAR $\gamma$  agonists are underway to treat diabetes, inflammation and cancer.<sup>39</sup> In this study we have analyzed the effect of endogenous n-3 FA and CR on the expression of PPAR $\gamma$  in the liver. No effect of CR on the expression of PPAR $\gamma$  was found. However, a significant increase in PPAR $\gamma$  expression was noted, due to the endogenous production of n-3 FA (Fig.

6). A slight but non-significant increase, in PPAR $\gamma$  expression was noted in the liver of Fat-1/CR mice, as compared to that of Fat-1/AL mice. However, a significantly higher level of PPAR $\gamma$  was observed in the liver of Fat-1/CR mice, as compared to that of WT/CR mice (Fig. 6).

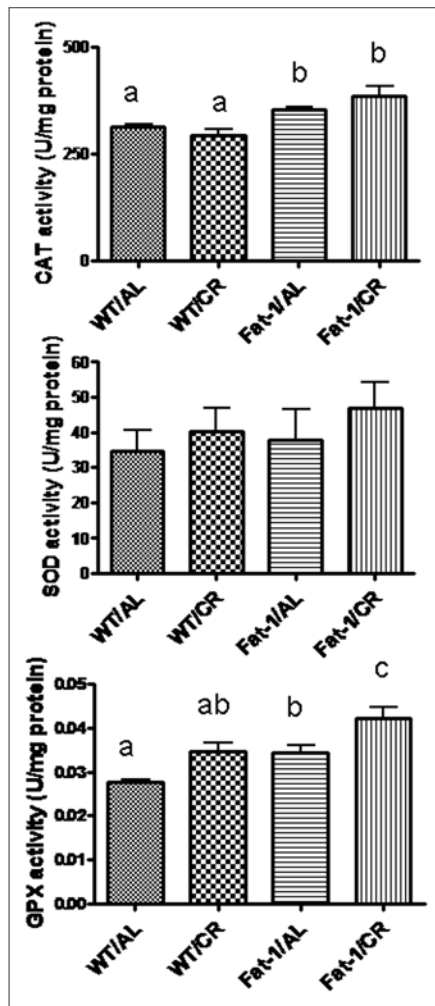
To determine the synergistic effect of endogenously synthesized n-3 fatty acids and CR, we have analyzed the data for different parameters by Two Way Anova using Graph Pad Prism software. We found synergistic effect of endogenously synthesized n-3 fatty acids and CR on the expression of muscle catalase, liver PPAR $\gamma$  and liver SIRT-1 (Table 1). We also found interactive effect although not significant, on some other parameters (Table 1).

## Discussion

The study presented here was designed to examine the effect of an endogenously increased n-3 PUFA status on the anti-inflammatory and anti-oxidative property of Fat-1 transgenic mice on a moderate CR diet. Significant reduction of pro-inflammatory cytokines production and augmentation of anti-oxidant enzymes production, along with enhanced expression of PPAR $\gamma$  and SIRT-1, were observed in Fat-1/CR mice. These findings suggest that endogenous n-3 FA and CR, in combination, may prevent or delay the progression of inflammation and oxidative stress related pathological consequences, thereby may prolong life span. In this study, we used Fat-1 transgenic mice, which were developed recently by Dr. Kang. These transgenic mice express a *C. elegans* desaturase, leading to the formation of high levels of n-3 PUFA endogenously from n-6 PUFA.<sup>28</sup> In this study, mice were fed a n-6 enriched and n-3 FA deficient diet which resembles western diet which contains a n-6 to n-3 FA ratio of 10:1-20:1.<sup>36</sup> Different tissues of Fat-1 mice show an increase in n-3 FA, leading to a significant decrease in n-6/n-3 FA ratio, compared to WT mice. In our present study, we also found increased n-3 FA and decreased n-6/n-3 FA ratio in serum, muscle and liver. Studies with Fat-1 mouse have already yielded interesting results and became a very valuable tool to study the effect of n-3 FA in health and diseases.<sup>2,29-34</sup> Compared to a FA feeding study, using Fat-1 transgenic mice require only one diet to be provided to all experimental mice groups, thereby free of potential confounders, like variations in calories with respect to fat content, sources or quality of different oils. Thus, the Fat-1 mouse represents a significant advance in the development of a more sophisticated research model to investigate the effect of n-3 FA and n-6/n-3 FA ratio on physiological parameters, inflammation, oxidative stress and molecular mechanisms, without providing exogenous n-3 FA in the form of fish oil (FO).

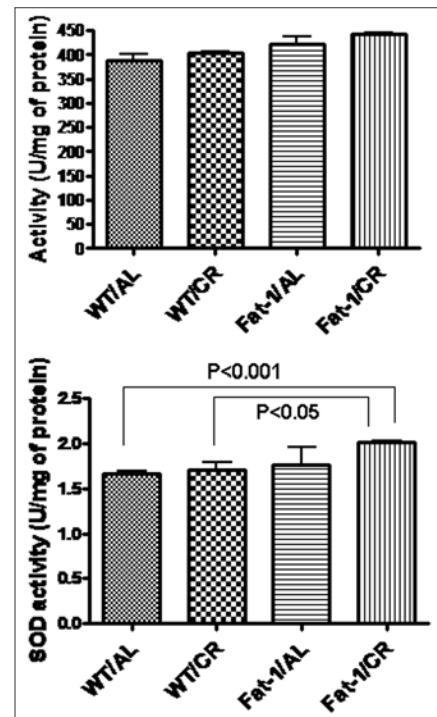
Inflammation is associated with aging and with various diseases. Anti-inflammatory properties of both n-3 FA and CR are established. Earlier, we have showed that n-3 FA prevents ovariectomy induced osteoporosis, age-associated osteoporosis and arthritis in animal models by attenuating the production of pro-inflammatory cytokines and inflammatory osteotropic factors.<sup>6,34,40,41</sup> Others have also showed that n-3 FA decrease pro-inflammatory cytokines production in tissues and cells.<sup>42</sup> Recent





**Figure 3.** Endogenous n-3 FA and CR enhance muscle anti-oxidant enzymes activity: After 5 months of dietary intervention, muscle homogenates were prepared and used to determine the activity of catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPX) as described in materials and methods section. Each bar represents the mean  $\pm$  SEM (n = 8-10/group). Value with different superscripts are significantly different at  $p < 0.05$  by Newman-Keuls' one way ANOVA. WT/AL, wild type ad libitum; WT/CR, wild type calorie restriction; Fat-1/AL, Fat-1 transgenic ad libitum; Fat-1/CR, Fat-1 transgenic calorie restriction.

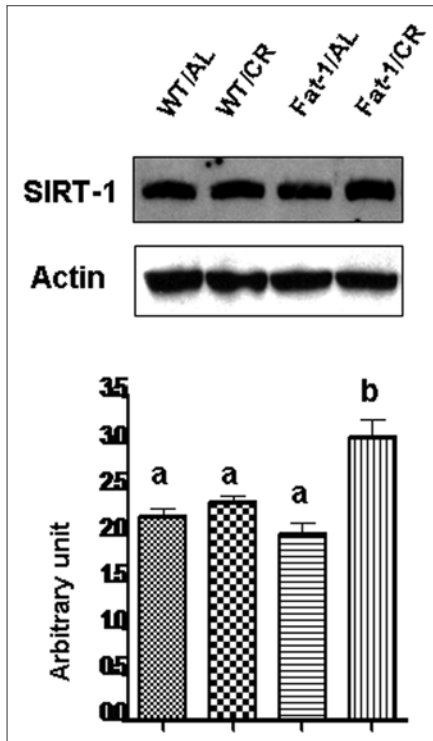
evidence has also documented CR's anti-inflammatory action, as shown in its modulation of pro-inflammatory genes, such as TNF $\alpha$ , IL-1, IL-6, c-reactive protein (CRP), adhesion molecules (AMs), iNOS and COX-2, through the NF $\kappa$ B signaling pathway.<sup>15,43</sup> In our previous study using Fat-1 mice fed 40% CR diet, we showed a synergistic effect of endogenous n-3 FA and CR on reducing pro-inflammatory cytokines production in LPS treated splenocytes. We showed that endogenous n-3 FA, in combination with CR, strongly inhibited the activation of two major intracellular inflammatory signaling pathways, NF $\kappa$ B and AP-1. Earlier, we and others have also reported that n-3 FA inhibit NF $\kappa$ B activation.<sup>6,29,34,42</sup> A synergistic anti-inflammatory effect of CR and n-3 FA has been recently reported by others.<sup>15</sup> In the present study, we found decreased serum IL-6, TNF $\alpha$  and IL-1 $\beta$  levels in Fat-1/



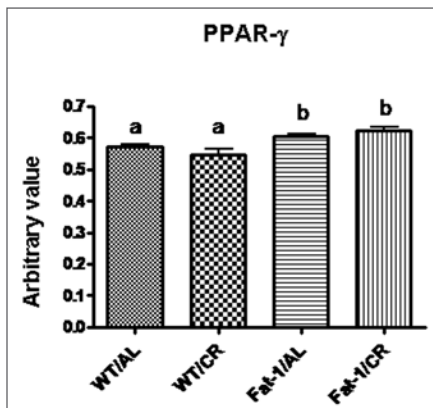
**Figure 4.** Endogenous n-3 FA and CR enhance liver anti-oxidant enzymes activity: After 5 months of dietary intervention, muscle homogenates were prepared and used to determine the activity of catalase (CAT), and superoxide dismutase (SOD) as described in materials and methods section. Each bar represents the mean  $\pm$  SEM (n = 8-10/group). The p values shown on SOD bars were determined by Student's t test. WT/AL, wild type ad libitum; WT/CR, wild type calorie restriction; Fat-1/AL, Fat-1 transgenic ad libitum; Fat-1/CR, Fat-1 transgenic calorie restriction.

AL mice, compared to WT/AL mice. A further stronger reduction of serum pro-inflammatory cytokines levels was observed in Fat-1/CR mice, as compared to that of WT/CR mice. This is in concurrence with our previous Fat-1/CR study, as well as other studies, with n-3 FA feeding.<sup>29,42</sup> It has also been reported that n-3 FA inhibited LPS-stimulated cytokine production in human mononuclear cells and THP-1 macrophages.<sup>44</sup> CR was reported to prevent age-related increase in circulating TNF $\alpha$  and IL-6 in long-lived C3B10RF1 mice,<sup>45</sup> in myocardial ischemia-reperfusion induced inflammation and other diseases.<sup>46</sup> In the present study, CR alone exhibited a reduction of serum IL-1 $\beta$  level, without significant reduction of serum IL-6 and TNF $\alpha$  levels. As seen in our previous Fat-1/CR study, a dramatic inhibition of serum TNF $\alpha$ , IL-6 and IL-1 $\beta$  was observed in Fat-1/CR mice, as compared to that of WT/CR mice. These data provide further evidence that n-3 FA and CR may have a synergistic anti-inflammatory effect. This is an important finding that warrants further investigations in order to determine the prophylactic and therapeutic effect of n-3 FA and CR combination on various inflammatory diseases. However, this dietary strategy may not be suitable for people living in pathogen-rich environments as the reduction of inflammatory cytokines will reduce immune system defenses.<sup>47-50</sup>

Oxidative stress is considered one of the major contributory factors of aging and also associated with a number of pathological



**Figure 5.** Endogenous n-3 FA and CR upregulate the level of sirtuin (SIRT)-1 expression in liver: After 5 months of dietary intervention, whole liver tissue extracts were prepared and performed western blot analysis for SIRT-1 protein levels in different experimental groups. The bottom lane indicates equal loading of proteins probed for actin antibody. Bars represent relative densitometric expression of SIRT-1. Each bar represents the mean  $\pm$  SEM (n = 4/group). Value with different superscripts are significantly different at  $p < 0.05$  by Newman-Keuls' one way ANOVA. WT/AL, wild type ad libitum; WT/CR, wild type calorie restriction; Fat-1/AL, Fat-1 transgenic ad libitum; Fat-1/CR, Fat-1 transgenic calorie restriction.



**Figure 6.** Endogenous n-3 FA and CR upregulate the level of peroxisome proliferator-activated receptor (PPAR) $\gamma$  expression in liver: After 5 months of dietary intervention, liver nuclear proteins were prepared and analyzed for PPAR $\gamma$  levels using TransAM PPAR $\gamma$  Activation Assay kit from Active Motif. Each bar represents the mean  $\pm$  SEM (n = 4/group). Value with different superscripts are significantly different at  $p < 0.05$  by Newman-Keuls' one way ANOVA. WT/AL, wild type ad libitum; WT/CR, wild type calorie restriction; Fat-1/AL, Fat-1 transgenic ad libitum; Fat-1/CR, Fat-1 transgenic calorie restriction.

conditions. There is evidence that oxidative stress and inflammation are closely related to each other. Where there is oxidative stress, inflammation is likely to occur. Inflammation and oxidative stress promote catabolic stimuli, such as, IL-6, IL-1 and TNF $\alpha$ . Oxidative stress occurs when the balance between free radicals and anti-oxidant enzymes favors toward the former. That means the more production of anti-oxidant enzymes, the less generation of oxidative stress. Both n-3 FA and CR are considered as anti-oxidative.<sup>8</sup> We and others have shown that n-3 FA enhance the anti-oxidant defense system of animals and humans by increasing the production of anti-oxidant enzymes.<sup>40,51,52</sup> The beneficial effect of CR in health and diseases is also thought to be due mainly to its powerful anti-inflammatory, resistance against oxidative stress and its ability to maintain a proper cellular redox status.<sup>15</sup> CR is also known to suppress COX-derived ROS generation.<sup>53</sup> Antioxidant defense system decreases with age and CR is shown to prevent these decreases.<sup>16</sup> Thus, the effect of CR is believed to be due to its powerful anti-oxidative action.<sup>15</sup> Animal studies have shown repeatedly that CR enhances metabolic efficiency, decreases inflammation and increases stress resistances over the lifespan by blunting the age-dependent increase of NF $\kappa$ B activation.<sup>15,54</sup> In this study, we found a higher level of CAT, SOD and GPX in muscle and CAT and SOD in liver of Fat-1 mice, as compared to that of WT mice. There was a further enhancement of this anti-oxidant enzymes production in Fat-1/CR mice, as compared to that of WT/CR. These data suggest that there is a synergistic effect of endogenous n-3 FA and CR on the production of anti-oxidant enzymes, thereby potentially enhancing the antioxidant defense system, reducing pathological conditions and therefore, may prolong life span.

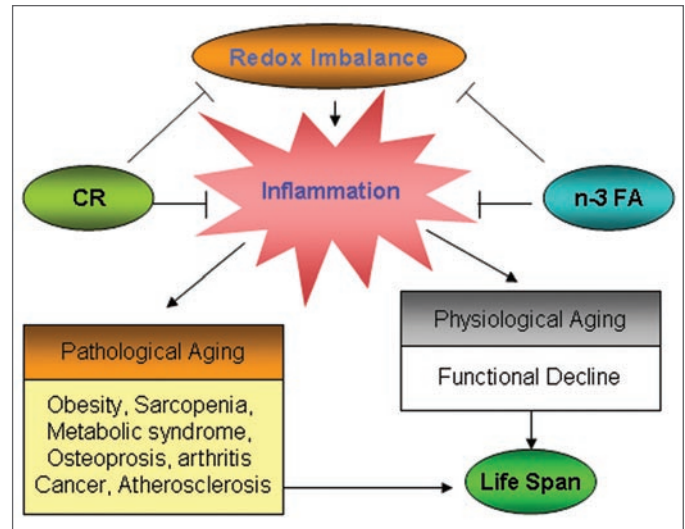
Both inflammation and oxidative stress increase with the progression of aging (Fig. 7). We previously demonstrated the life-prolonging effects of n-3 FA in NZB/W mouse model of human SLE, as compared to n-6 FA.<sup>14</sup> When n-3 FA was combined with 40% CR, these mice lived significantly longer compared to n-6 FA + CR.<sup>55</sup> Recently, life prolonging genes have been discovered and named sirtuins (SIRT1-7 in humans), which are anti-inflammatory in nature.<sup>56</sup> It has been demonstrated that CR increases the expression of SIRT-1.<sup>38</sup> SIRT1 is likely to directly regulate the transcriptional activity of NF $\kappa$ B.<sup>57</sup> Activation of SIRT-1 induced by CR would also explain the resistance to inflammation that occurs during CR via the inhibition of NF $\kappa$ B signaling. Microarray studies have revealed that the age-related pro-inflammatory gene expression profiles can be reprogrammed via CR.<sup>58</sup> It has been also reported that SIRT-1 attenuates oxidative stress-induced mesangial cell apoptosis via p53 deacetylation.<sup>59</sup> Sklavounou et al. have reported that SIRT-1 plays an important role in preventing differentiation and apoptosis as well as increasing cellular resistance to stress.<sup>60</sup> It has also been shown that overexpression of SIRT-1 enhances neuronal survival by preventing nuclear DNA degradation during oxidative stress.<sup>20,61,62</sup> Activation of SIRT-1 was also shown to be associated with inhibition of NF $\kappa$ B activation and oxidative stress.<sup>63</sup> In this study, we found an upregulation of SIRT-1 expression in Fat-1/CR mice, as compared to that of WT/CR mice. However, endogenous n-3 FA did not modulate the expression of SIRT-1. CR alone did increase

**Table 1.** Statistical determination of the combined effect of endogenously synthesized n-3 fatty acids (FA) and calorie restriction (CR) on different parameters

Parameters	Gene	Diet	Gene X diet
Serum n-6/n-3 FA ratio	<0.0001	0.0753	0.4089
Muscle n-6/n-3 FA ratio	<0.0001	0.2847	0.3878
Liver n-6/n-3 FA ratio	<0.0001	0.4705	0.6884
Serum TNF $\alpha$	0.0011	0.0422	0.9157
Serum IL-6	<0.0001	0.0346	0.5213
Serum IL-1 $\beta$	0.0006	<0.0001	0.3330
Muscle CAT	<0.0001	0.740	0.0482
Muscle SOD	0.5022	0.3324	0.7935
Muscle GPX	0.0022	0.0017	0.7390
Liver CAT	0.0041	0.1114	0.6960
Liver SOD	0.1410	0.2802	0.4146
Liver SIRT-1	0.0671	0.0004	0.0035
Liver PPAR $\gamma$	<0.0001	0.4350	0.0215

Values represent the p values by Two Way Anova using Graph Pad Prism software. p value <0.05 is considered significant. Gene: Fat-1 gene capable of converting n-6 FA to n-3 FA. Diet: ad libitum and calorie restriction (40% less than ad libitum). TNF $\alpha$ , tumor necrosis factor- $\alpha$ ; IL-6, interleukin-6; IL-1 $\beta$ , interleukin-1  $\beta$ ; CAT, catalase; SOD, superoxide dismutase; GPX, glutathione peroxidase; SIRT-1, sirtuin-1; PPAR $\gamma$ , peroxisome proliferator-activated receptor- $\gamma$ .

SIRT-1 expression, but remarkable augmentation of SIRT-1 was observed when combined with endogenous production of n-3 FA, demonstrating that combination of n-3 FA and CR may extend life span even further than CR alone. Thus, combination of n-3 FA and CR may inhibit inflammation and oxidative stress by enhancing the expression of SIRT-1. PPAR $\gamma$  is a master regulator of glucose, insulin and lipid metabolism<sup>64,65</sup> and n-3 fatty acid and CR are known to increase PPAR $\gamma$  mRNA and protein expression.<sup>54</sup> PPAR $\gamma$  is also anti-inflammatory by nature.<sup>66</sup> CR is shown to attenuate age-related decreases of PPARs, thus boosting the inhibitory effect of PPARs on NF $\kappa$ B activation. Further, PPAR $\gamma$  agonists are reported to inhibit inflammatory cytokine production and PPAR $\gamma$  is downregulated by inflammatory cytokines.<sup>39,67</sup> Moreover, the evidence to date suggests that activation of PPAR $\gamma$  should suppress tumor growth and development.<sup>39</sup> Similar to SIRT-1, very recently, overexpression of PPAR $\gamma$  has been shown to be associated with increased resistance against oxidative stress.<sup>24-27</sup> Moreover, n-3 FA deficiency related hepatic steatosis involves reduced protection against endoplasmic reticulum stress due to reduced expression of PPAR $\gamma$ .<sup>68</sup> Chung et al. reported that in human skeletal muscle cells, the antioxidant effect of PPAR $\gamma$  is exclusively mediated by glutathione peroxidase.<sup>69</sup> It has also been shown that increased expression of PPAR $\gamma$  and SOD1 is associated with decreased accumulation of oxidized LDL.<sup>70</sup> In this study, we found upregulation of PPAR $\gamma$  expression in liver of Fat-1 mice, as compared to that of WT mice. There was further augmentation of PPAR $\gamma$  expression in Fat-1/CR mice, as compared to WT/CR mice, demonstrating the synergistic effect of n-3 FA and CR on the expression of PPAR $\gamma$ . These data suggest that upregulation of PPAR $\gamma$  expression may be one of the



**Figure 7.** The role of inflammation and oxidative stress in pathophysiological process and the possible action of CR and n-3 FA.

mechanisms by which the n-3 FA and CR exert its anti-inflammatory and anti-oxidative capacity. Furthermore, by inducing the expression of PPAR $\gamma$ , n-3 FA and CR also will improve the glucose, insulin and lipid metabolism, thereby improving the quality of life. These are very important new findings, which warrant urgent study in order to determine the combined effect of n-3 FA and CR on the prolongation of life span. Implementation of further work to elucidate the cellular mechanisms of n-3 FA and CR mediated anti-inflammatory and anti-oxidative activities in animals and humans may offer novel dietary approaches for the treatment of disorders tied to inflammation and oxidative stress as well as for the extension of lifespan.

In conclusion, the present data demonstrate that n-3 FA and CR, in combination, may improve the overall pathological conditions, by modulating inflammatory response and oxidative stimuli and associated intracellular signaling molecules, with the progression of age, thereby improving the quality of aging and may extend the lifespan.

## Materials and Methods

**Animals and experimental diets.** Male transgenic Fat-1 C57BL6 mice were obtained from Dr. Jing Kang at the Harvard Medical School. They were mated with wild type C57BL6 female mice to obtain female *fat-1* positive C57BL6 mice (Fat-1) and *fat-1* negative C57BL6 mice (WT) identified by genotyping using REDExtract-N-Amp Tissue PCR Kit from Sigma and analyzing the fatty acid composition of tails by using gas chromatography (GC) as described previously.<sup>29</sup> Weight-matched mice were housed in a laboratory animal care facility in cages (3–4 mice/cage) and fed semi-purified AIN-93M diets containing 10% corn oil (CO) (MP Biomedicals, Irvine, CA). CO is high in linoleic acid (18:2n-6) and Fat-1 mice convert n-6 FA to n-3 FA. The composition of the semi-purified diet per kilogram of diet was: 140 g of casein, 424.3 g of corn starch, 145 g of dextrinized corn



starch, 90 g of sucrose, 50 g of fiber, 35 g of AIN-93 mineral mix, 10 g of AIN-93 vitamin mix, 1.8 g of l-cystine and 2.5 g of choline bitartrate. Diets were prepared weekly and stored in aliquots at -20°C. Fresh diet was provided daily, and leftover food was removed to prevent rancidity. 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. At the end of experimental diet, body weights measured for WT/AL, WT/CR, Fat-1/AL and Fat-1/CR groups were 36.54 ± 2.55 g, 23.70 ± 0.66 g, 33.90 ± 3.30 g and 25.74 ± 1.30 g respectively. The National Institutes of Health guidelines provided in “The Guide for the Care and Use of Laboratory Animals” were strictly followed, and all studies were approved by the Institutional Laboratory Animal Care and Use Committee of the University of Texas Health Science Center at San Antonio.

**Collection of blood serum.** At the termination of the study, blood was collected and serum was obtained by centrifugation at 300 g for 15 min at 4°C.

**Serum cytokine measurement.** Serum cytokine levels for TNF $\alpha$ , IL-6 and IL-1 $\beta$  were measured by standard enzyme-linked immunosorbent assay (ELISA) techniques using commercially available BD OptEIA ELISA kits for TNF $\alpha$ , IL-6 and IL-1 $\beta$  (BD Biosciences, San Diego, CA), as described previously.<sup>51</sup> Each well of flat-bottom 96-well microtiter plates was coated overnight with 100  $\mu$ l of purified anti-TNF $\alpha$ , anti-IL-6 or anti-IL-1 $\beta$  antibodies (4  $\mu$ g/ml in binding solution) at 4°C. The plates were rinsed four times with washing buffer, and then samples were added, followed by incubation for 2 h at room temperature. The plates were washed four times with washing buffer, followed by the addition of biotinylated anticytokine antibodies. The plates were incubated in room temperature for 1 h and then washed four times with washing buffer. Streptavidin–alkaline phosphatase conjugate was added, and the plates were incubated for 30 min at room temperature. The plates were again washed four times with washing buffer, and chromogen substrate was added. The plates were then incubated at room temperature to achieve the desired maximum absorbance and were read at 410 nm in an ELISA reader (Dyner Technologies, UK).

**Measurement of anti-oxidant enzymes activity in muscle and liver.** The muscles (gastrocnemius and quadriceps) and liver tissues obtained from different experimental groups were homogenized in 2 ml tris-HCl buffer (pH 7.4) and sonicated for 16 s. Thereafter, the homogenates were centrifuged (10,000 g for 2 min) and the supernatant were used for the estimation of SOD, CAT and GPX, as described previously.<sup>40,51</sup> The protein content of spleen supernatants was determined by the microplate procedure of the bicinchoninic acid (BCA) protein assay as described

by the supplier (Pierce Chemical Company, Rockford, IL). SOD activity: assay was based on the ability of SOD to inhibit the spontaneous oxidation of adrenaline to adrenochrome. Results are expressed as units (U) of SOD activity/mg protein. One unit of SOD induced approximately 50% inhibition of auto-oxidation of adrenaline. CAT activity was measured based on the ability of CAT to induce the disappearance of hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), which was followed spectrophotometrically.<sup>71</sup> One unit (U) was defined as the amount of the enzyme required to decompose 1 mM of H<sub>2</sub>O<sub>2</sub> per min, at 25°C and pH 7.0. Results are expressed as units (U) of CAT activity/mg protein. GPX activity: H<sub>2</sub>O<sub>2</sub> was used as the substrate. Sodium azide (1 mM) was added to the reaction mixture to inhibit remnant CAT activity. One unit of GPX was defined as the amount of the enzyme decomposing 1 mol H<sub>2</sub>O<sub>2</sub> per min, at 25°C and pH 7.0. Results are expressed as units (U) of GPX activity/mg protein.

**Preparation of liver homogenates.** Cytosolic and nuclear extracts of liver were prepared using a Nuclear Extract Kit from Active Motive (Carlsbad, CA), following manufacturer’s instruction. Whole liver tissue extracts were prepared using a lysis buffer containing 10 mM Tris HCl (pH 7.8), 1% Nonidet P40 (NP40), 0.15 M NaCl, 1 mM EDTA, 1 mM DTT, 0.1% Triton X, 0.2 mM AEBSF, 50 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub> and protease inhibitor cocktail from Roche Diagnostics. 100 mg of liver tissues were homogenized and briefly centrifuged to separate debris, then kept on ice for 30 minutes, then centrifuged at 12,000 rpm for 20 minutes at 4°C and collected supernatant as whole liver tissue extract.

**Western blot analysis for SIRT-1.** 50  $\mu$ g of whole liver tissue extracts were subjected to SDS-PAGE. Proteins were transferred to immunoblot nitrocellulose membranes (BioRad, Hercules, CA) and subjected to western blot analysis to determine the level of SIRT-1 in different experimental groups. Polyclonal antibody against SIRT-1 and monoclonal antibody against actin obtained from Santa Cruz Biotechnology Inc., were used. Western blot was performed as described earlier.<sup>72,73</sup>

**Analysis of PPAR $\gamma$  in liver.** Nuclear extracts of liver samples were used to determine the level of PPAR $\gamma$  using TransAM PPAR $\gamma$  Activation Assay Kit (Cat # 40196) from Active Motif, following manufacturer’s instruction.

**Statistical analysis.** Data are expressed as means  $\pm$  SEM. To test the significance either student’s t-test or Newman-Keuls’ one way ANOVA was used. A p value  $\leq$ 0.05 was considered statistically significant. The analyses were performed using Graphpad prism for Windows (La Jolla, CA, USA).

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