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n-3 and n-6 polyunsaturated fatty acids differentially regulate adipose angiotensinogen and other inflammatory adipokines in part via NF-κB-dependent mechanisms

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Abstract

Excessive secretion of proinflammatory adipokines has been linked to metabolic disorders. We have previously documented anti-inflammatory effects of n-3 polyunsaturated fatty acids (n-3 PUFAs) in adipose tissue; however, the mechanisms by which these fatty acids regulate adipokine secretion remain unclear. Here, we determined differential effects of eicosapentaenoic acid (EPA, n-3 PUFA) vs. arachidonic acid (AA, n-6 PUFA) on expression and secretion of angiotensinogen (Agt), interleukin 6 (IL-6) and monocyte chemotactic protein (MCP-1) in 3T3-L1 adipocytes. While both PUFAs increased intracellular Agt protein and mRNA expression, Agt secretion into culture media was increased only by AA treatment, which in turn was prevented by co-treatment with EPA. At various AA/EPA ratios, increasing AA concentrations significantly increased secretion of the above three adipokines, whereas increasing EPA dose-dependently, while lowering AA, decreased their secretion. Moreover, IL-6 and MCP-1 were more significantly reduced by EPA treatment compared to Agt (IL-6>MCP>Agt). Next, we tested whether nuclear factor-κB (NF-κB), a major proinflammatory transcription factor, was involved in regulation of these adipokines by PUFAs. EPA significantly inhibited NF-κB activation compared to control or AA treatments. Moreover, EPA attenuated tumor necrosis factor-α-induced MCP-1 and further reduced its secretion in the presence of an NF-κB inhibitor. Taken together, we reported here novel beneficial effects of EPA in adipocytes. We demonstrated direct anti-inflammatory effects of EPA, which are at least in part due to the inhibitory effects of this n-3 PUFA on the NF-κB pathway in adipocytes. In conclusion, these studies further support beneficial effects of n-3 PUFAs in adipocyte inflammation and metabolic disorders.

Keywords: PUFA; Arachidonic acid; Eicosapentaenoic acid; Adipocyte; Inflammation; MCP-1; IL-6; Agt

1. Introduction

Obesity increases the risk for metabolic, inflammatory and cardiovascular diseases (CVDs) [1]. The severity of obesity-related health consequences is further exacerbated by its burden on health care with yearly medical costs of obesity well exceeding $100 billion [1,2].

Obesity is a major contributor and an independent risk factor for insulin resistance, diabetes and CVDs [3–6] and one of the most important risk factors for the development of hypertension [6]. Chronic, low-grade inflammation is now recognized as the basis for several metabolic disorders and may thus further worsen obesity-related consequences [7,8]. Therefore, studies which focus on understanding the mechanisms associated with obesity-related risk for inflammation and its prevention can potentially impact overall disease prevention and treatment.

Adipose tissue is now well established as a dynamic endocrine organ which secretes a variety of pro- and anti-inflammatory cytokines termed adipokines [9]. An imbalance in production and/or secretion of these peptides contributes to the development of obesity-related disorders via paracrine, autocrine and endocrine effects [10,11]. Thus, we addressed whether adipose tissue secretion of angiotensinogen (Agt) and other proinflamatory cytokines is regulated by dietary fatty acids with known anti-inflammatory properties. This study focused on the regulation of Agt, interleukin-6 (IL-6) and monocyte chemotactic protein-1 (MCP-1), major adipokines linked to inflammation and associated metabolic alterations. Agt is the only

Abbreviations: Agt, angiotensinogen; Ang II, angiotensin; PUFA, polyunsaturated fatty acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; NF-κB, nuclear factor-κB; IL-6, interleukin-6; MCP-1, monocyte chemotactic protein-1.

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known precursor for the hypertensive hormone angiotensin II (Ang II). We and others have documented expression of Agt and other components of the renin angiotensin system (RAS) in adipose tissue in several species [12–14]. Further, contribution of adipocyte-derived Agt to systemic Agt levels was demonstrated in transgenic mice overexpressing Agt in adipose tissue [12,14]. The transgenic mice expressing Agt in adipose tissue exhibit both increased adiposity and hypertension, indicating an important role of the adipocyte specific RAS activation pathway [12]. In addition, recent studies have suggested that RAS might be associated with metabolic disorders and insulin resistance, while RAS inhibition improved the metabolic profile and insulin sensitivity [15–18]. Specifically, targeted inactivation of components of RAS [Agt, angiotensin-converting enzyme (ACE), renin, angiotensin type 1 or type 2 receptors] in animal models or pharmacological blockade of RAS by angiotensin II receptor blockers or ACE inhibitors reduced fat cell size and prevented diet-induced obesity and insulin resistance [15–20]. Also, Ang II exerts lipogenic effects in cultured murine cells [21,22].

Consequently, these studies implicate Ang II as a potentially significant factor in the development of obesity-related hypertension and insulin resistance in conditions of expanded adipose mass. Moreover, inflammation has been documented as the basis for several chronic diseases. Agt and other proinflammatory adipokines such as IL-6 and MCP-1 are secreted from adipose tissue in an obesity-dependent manner [6–11]. IL-6 is a proinflammatory cytokine expressed in adipose tissue and known to induce insulin resistance [23]. MCP-1 can trigger macrophage infiltration and has also been associated with insulin resistance [24,25]. Moreover, increased gene expression of several proinflammatory cytokines including MCP-1, IL-6 and tumor necrosis factor (TNF)-α has been shown to be induced when nuclear factor (NF)-κB is activated [25]. Further, activation of NF-κB in adipocytes and macrophages contributes to chronic inflammation in obese individuals [26,27].

Beneficial effects of n-3 PUFAs in CVD as hypolipidemic agents are well documented [28]. However, less is known about its role in modulating adipocyte biology and endocrine and inflammatory function. Omega-3 (n-3) polyunsaturated fatty acids (n-3 PUFAs) such as eicosapentaenoic acid (EPA) are anti-inflammatory nutrients known to suppress the activation of NF-κB in several tissues [29]. We and other investigators have previously shown that n-6 PUFAs increase the secretion of proinflammatory substances such as prostaglandins (PGE_2) in adipocytes and that n-3 PUFAs counteracted these effects [30–34]. Therefore, we hypothesized that n-3 PUFA can decrease secretion of Agt and other inflammatory cytokines from adipocytes and that these effects are at least partially due to reduced NF-κB activation in adipocytes.

Given this central role for adipose tissue inflammation in obesity-associated metabolic syndrome, we propose that n-3 PUFA is a potential target for alleviating metabolic inflammatory disorders. Further, because of well-established competitive effects of n-3 and n-6 PUFAs, both for incorporation in tissue membranes and for eicosanoid biosynthetic enzymes [28–30], we specifically tested whether the n-3 PUFA EPA down-regulates expression and/or secretion of Agt, IL-6 and MCP-1 at various arachidonic acid (AA)/EPA ratios. We demonstrate that EPA significantly decreased secretion of all three adipokines, an effect that was attenuated by increasing amounts of AA. Further, these beneficial effects of EPA were at least in part via inhibition of NF-κB.

2. Materials and methods

2.1. Cells and reagents

3T3-L1 preadipocytes were purchased from American Type Culture Collection (ATCC, Rockville, MD, USA). Cell culture media and other reagents were purchased from GIBCO (Carlsbad, CA, USA) and Sigma-Aldrich Corp. (St. Louis, MO, USA). Fatty acids were purchased from Nu-Check Prep, Inc. (Elysian, MN, USA). Agt antibody was purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). TNF-α (Promega, Madison, WI, USA) and BAY-11-7082 (Calbiochem, San Diego, CA, USA) were prepared according to the manufacturers' guidelines.

2.2. 3T3-L1 cell culture

3T3-L1 mouse embryonic preadipocytes were cultured as we previously described [34]. Briefly, 3T3-L1 cells were cultured in regular growth media consisting of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin (P/S). Confluent cells were differentiated into adipocytes by treatment with 0.5 mM 1-methyl-3-isobutylxanthine, 0.25 μM dexamethasone and 10 nM insulin (Humulin R, Eli Lily) for 2 days and then were maintained in regular growth media. Differentiation was considered complete 5–7 days after confluence with ~80%–95% of the cells being differentiated. Twenty-four hours prior to fatty acids or other treatments, growth media were changed to starvation media consisting of serum-free DMEM with antibiotics, 1% (wt/vol) fatty acid-free bovine serum albumin (BSA) (Sigma-Aldrich, St. Louis, MO, USA), 10 nM insulin and 1% P/S.

2.3. Fatty acid treatments

Differentiated cells were treated with varying concentrations of EPA and AA, as we previously described [34]. Briefly, the fatty acids were reconstituted in dimethyl sulfoxide (DMSO) and then conjugated to fatty-acid-free BSA by agitation in a 37°C water bath for 2 h prior to treatments. Fatty acid treatment media consisted of serum-free DMEM with antibiotics, 1% fatty-acid-free BSA, 10 nM insulin and vehicle or the fatty acids, as described in the results and figures. Following 48 hr of treatment, cells and culture media were harvested and stored at −80°C for future use.

2.4. Mice and diets

Male C57BL/6J mice were obtained from the Jackson Laboratory. Following a 1-week acclimation period, they were given a high-fat (HF) diet (45% energy from fat; Research Diets) for 6 weeks. Mice were subsequently either continued on the same diet (HF group) or switched on to an HF-EPA diet (36 g/kg EPA ethyl ester; 45% energy from fat) for an additional 5 weeks. At the end of the study period, mice were feed-deprived for 4 h and killed by CO_2 inhalation. Epididymal adipose tissue was dissected out, snap-frozen in liquid N_2 and stored at −80°C for subsequent analysis. Further details of the mice and detailed composition of diets have been previously described [35].

2.5. Proinflammatory cytokine assays

Mouse multiplex cytokine/adipokine kits were purchased from Millipore (Bedford, MA, USA), and assays were performed according to the manufacturer's guidelines. Mouse multiplex adipokine kit contained monocyte chemotactic protein-1 (MCP-1), leptin, interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), total plasminogen activator inhibitor-1 (PAI-1), and adiponectin and was used to assay cytokines from the *in vitro* studies. Total proteins from the epididymal adipose tissue were isolated by homogenizing in the modified radioimmunoprecipitation buffer containing a cocktail of protease inhibitors. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to assay Agt (IBL, Minneapolis, MN, USA), Ang II (SPI-bio, Montigny le Bretonneux, France) and IL-6 (Invitrogen Corporation, Carlsbad, CA, USA). Analyte levels were normalized for total protein content.

2.6. Western immunoblotting

Total proteins were isolated from cultured adipocytes by homogenizing cells with modified radioimmunoprecipitation assay buffer containing a cocktail of protease inhibitors. Equal total amount of proteins were loaded (20 μg) into each lane and resolved by electrophoresis. Proteins were then transferred to a nitrocellulose membrane and blocked with 5% milk. These membranes were next incubated with primary antibodies of interest and respective secondary antibodies for detection. Expression levels were quantified using ImageJ software from National Institutes of Health. Samples of conditioned culture media from each treatment were directly mixed with sample buffer prior to loading.

2.7. NF-κB reporter gene activity measurements

3T3-L1 cells stably transfected with NF-κB responsive reporter (NF-κB-Luc, luciferase reporter, kindly provided by Dr. D. Hwang) were cultured, differentiated and treated as described above for 3T3-L1 cells. Following treatments indicated in the Figures and Results section, cells were harvested, and luciferase reporter assays were conducted using a luciferase assay from Promega (Madison, WI, USA). Briefly, the treated cells were washed three times with phosphate-buffered saline and lysed with the lysis buffer provided by the manufacturer. An aliquot of 50 μl was added into a white-bottom 96-well plate and mixed with 80 μl of Luc assay reagent. Luciferase activity was measured by GloMax, a microplate multimode reader (Promega, Madison, WI, USA).

2.8. Statistical analyses

Data for Figs. 1, 2, 4 and 5 were analyzed using SAS software (version 9.2). Experimental results are reported as means±S.D. Analysis of variance test was used to compare overall group means. A significant overall *F* test was followed by post hoc comparisons using the least significant difference multiple comparisons procedure. The level of significance was defined as $P<.05$ for all tests. Data for Fig. 3 was analyzed using Student's *t* test. Differences were significant at $P<.05$.

3. Results

3.1. Agt expression is differentiation-dependent and regulated by PUFA in 3T3-L1 adipocytes

We assayed Agt secretion in the culture media from nondifferentiated preadipocytes and differentiated 3T3-L1 adipocytes using Western immunoblotting. Agt was detectable only in media from differentiated mature adipocytes, indicating that only mature adipocytes but not preadipocytes secrete Agt (Fig. 1A).

Treatment of differentiated adipocytes with AA significantly ($P<.05$) increased the secretion of Agt, while EPA did not change Agt secretion levels from adipocytes (Fig. 1B). Further, treatment of adipocytes with both AA and EPA significantly reduced Agt secretion compared to AA treatment alone, indicating the ability of EPA to prevent AA-induced adipose Agt secretion. Interestingly, both AA and EPA increased the intracellular Agt protein levels (Fig. 1C). Consistent with changes in Agt intracellular protein levels, treatment with AA significantly increased Agt mRNA expression compared to the control (Fig. 1D). However, treatment with EPA alone or combined AA and EPA did not significantly alter Agt mRNA levels compared to the control.

3.2. Regulation of Agt, IL-6 and MCP-1 secretion by EPA and AA

To gain further insight into the differential effects of n-3 and n-6 PUFAs on adipose inflammation and to gain insight into potential mechanisms mediating beneficial effects of incorporating EPA in the diet, we investigated effects of various AA and EPA dose combinations on Agt, IL-6 and MCP-1 secretion. Differentiated adipocytes were treated with different AA and EPA combinations/-ratios as follows [EPA (μM):AA (μM); 0:200, 50:150, 100:100, 150:50 and 200:0]. Overall, EPA did not alter Agt secretion compared to control, while AA increased Agt secretion. Moreover, increasing the EPA/AA ratio dose-dependently decreased Agt secretion (Fig. 2A). Similarly, increasing the EPA/AA ratio significantly decreased MCP-1 and IL-6 secretion (Fig. 2B and C). Although EPA itself did not alter Agt secretion compared to the control, EPA alone clearly reduced both IL-6 and MCP-1 secretion. Moreover, this effect was dose-dependent as down-regulation of these cytokines by EPA was more pronounced when EPA gradually substituted AA, and overall EPA effects were more pronounced on IL-6 and MCP-1 compared to Agt.

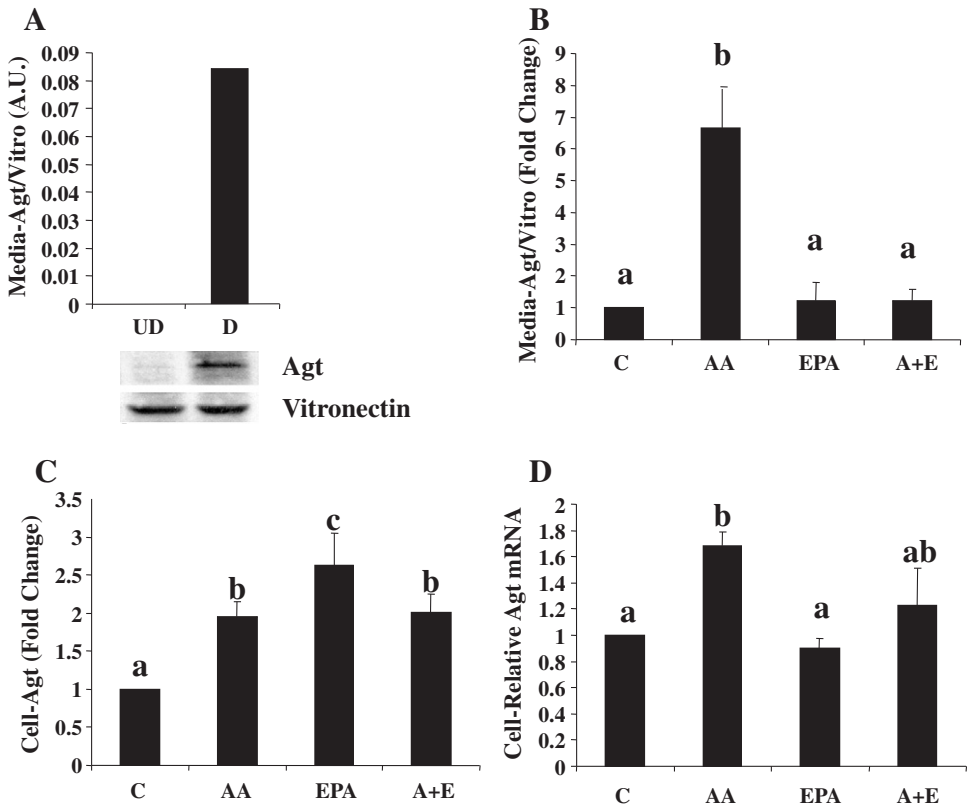

Fig. 1. Differential regulation of Agt protein and mRNA by adipocyte differentiation and PUFAs. 3T3-L1 cells were grown to subconfluence or differentiated into adipocytes as described in the Methods section. Culture media (A) were obtained from 2-day-old subconfluent undifferentiated (UD) 3T3-L1 preadipocytes or from 8-day-old differentiated (D) mature adipocytes; a representative Western blot is shown for Agt with vitronectin used as control. (B) Differentiated 3T3-L1 adipocytes were treated with vehicle (control, C), AA (150 μM), EPA (150 μM) or A+E (75 μM each of AA and EPA). Culture media were collected 48 h post PUFA treatments. Cell lysates from the same experiments were collected for intracellular Agt protein content (C) and Agt mRNA levels (D) measured by ELISA or real-time reverse transcriptase polymerase chain reaction, respectively. All assays were done in three biological triplicates for each set of data (i.e., three independent experiments). Results represent the mean±S.E.M. Values labeled with different letters are significantly different ($P<.05$). Values with the same letters do not differ significantly.

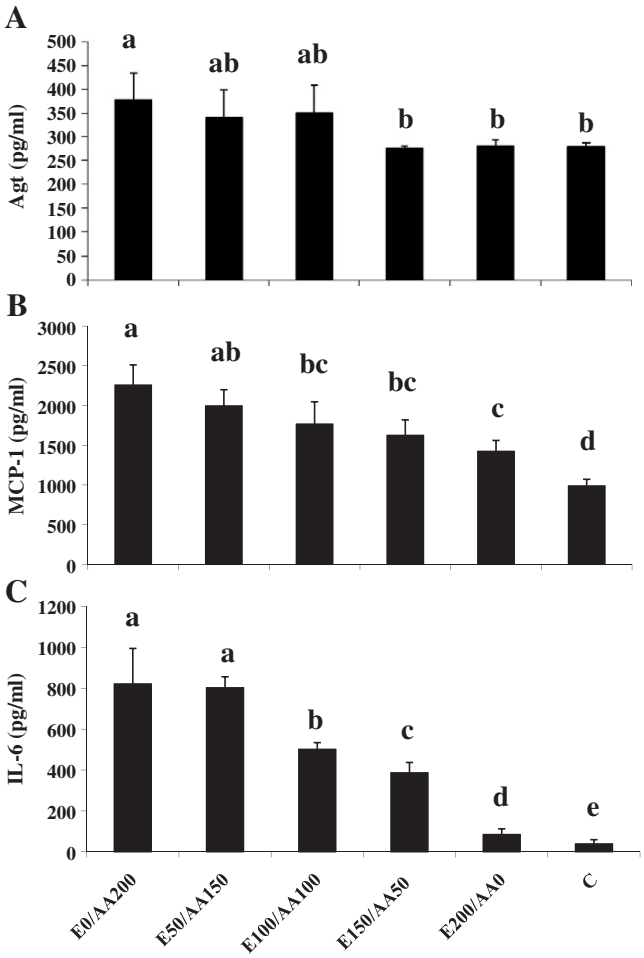

Fig. 2. Differential regulation of adipokines by PUFA. Differentiated adipocytes were treated with different AA and EPA ratios AA and EPA ratios; E is EPA and AA is Arachidonic Acid; numbers indicate concentrations in μM and C indicates the control/vehicle treatment. Culture media were collected after 48 h of PUFA treatment, and secreted Agt (A), MCP-1 (B) and IL-6 (C) were measured as described in the Methods section. Results represent the mean±S.E.M. Values labeled with different letters are significantly different ($P<.05$). Values with the same letters do not differ significantly.

3.3. EPA overcomes HF-diet-induced production of Ang II and IL-6 in epididymal adipose tissue of C57BL/6J mice

To investigate whether EPA produces similar changes in Agt and other cytokines *in vivo*, we studied levels of these cytokines in epididymal adipose tissue of mice continuously fed an HF diet for 11 weeks vs. mice fed an HF diet for 6 weeks followed by an HF-EPA diet for an additional 5 weeks. Detailed description of these diets and this research design has been recently published [35]. Mice fed EPA-supplemented HF diets exhibited significantly reduced Ang II and IL-6 levels in epididymal adipose tissue compared to mice fed HF diet without EPA (Fig. 3), despite having similar body and fat pad weights. However, no changes were noted in Agt levels in response to EPA treatment.

3.4. Effects of AA and EPA on NF-κB activation

Our studies above demonstrate that AA and EPA modulate adipokine secretion. NF-κB activation is a major mediator in inflammation which induces transcription of several inflammatory genes. Accordingly, we tested whether effects of these fatty acids were mediated by NF-κB using 3T3-L1 adipocytes stably transfected with an NF-κB-luciferase reporter construct (NF-κB-Luc). Increasing EPA/AA ratios dose-dependently decreased NF-κB activation. At 200 μM, EPA treatment significantly reduced NF-κB activity to levels below controls (vehicle-treated adipocytes) (Fig. 4). To further confirm these effects of EPA and AA on NF-κB activity, we co-treated 3T3-L1-NF-κB-Luc adipocytes with EPA with either a known NF-κB activator (TNF-α) or an NF-κB inhibitor (BAY 11-7085) to test their effects on Agt, IL-6 and MCP-1 secretion. Treatment of these cells with TNF-α dose-dependently increased the NF-κB activity, while treatment of BAY 11-7085 dose-dependently decreased the NF-κB activity (data not shown). TNF-α-induced NF-κB activation significantly increased the secretion of MCP-1 at both 1-nM and 10-nM TNF-α concentrations (Fig. 5A). EPA significantly reduced TNF-α-induced MCP-1 secretion at 1-nM TNF-α treatment but was unable to suppress MCP-1 secretion induced by 10-nM TNF-α treatment. Moreover, EPA significantly reduced TNF-α-induced Agt secretion at a dose of 0.1 nM TNF but not at higher TNF-α doses (data not shown). Additionally, co-treatment of EPA and BAY 11-7085 did not further reduce Agt secretion compared to untreated control levels or BAY 11-

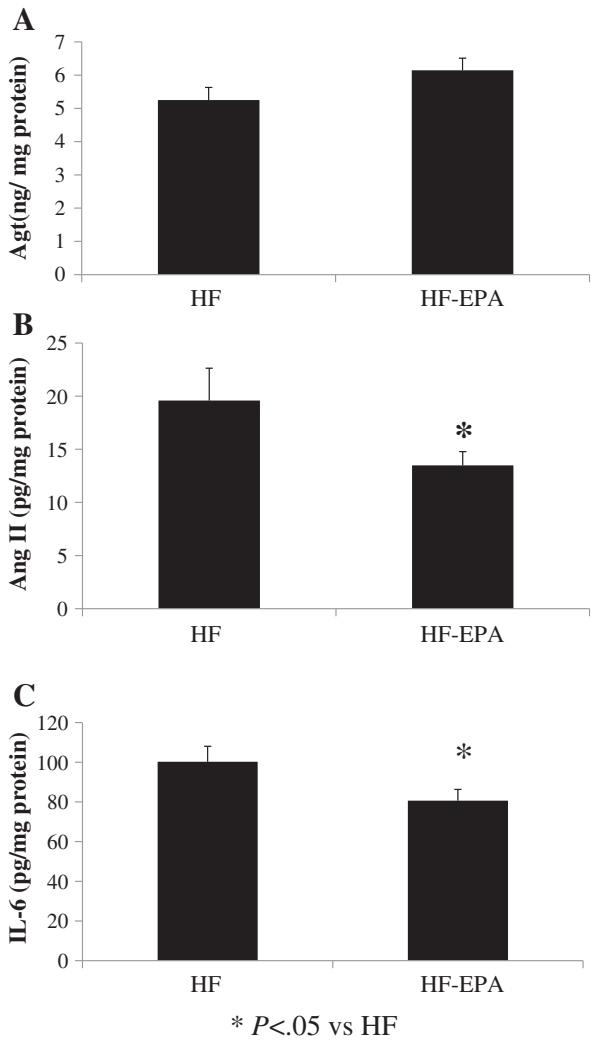

Fig. 3. Effects of EPA on adipose Agt, Ang II and IL-6 levels in HF-fed mice. Male C57BL/6J mice were fed an HF diet (45% energy from fat) for 11 weeks or HF diet for 6 weeks followed by an HF-EPA diet (36 g/kg EPA ethyl ester; 45% energy from fat) for 5 weeks. Epididymal adipose tissue was collected, and adipokine content was assayed. Adipose tissue Agt (A), Ang II (B) and IL-6 (C) levels are shown. Data are presented as mean ±S.E.M. (n=8–9 per group). Student's t test was used in the analysis at $P<.05$.

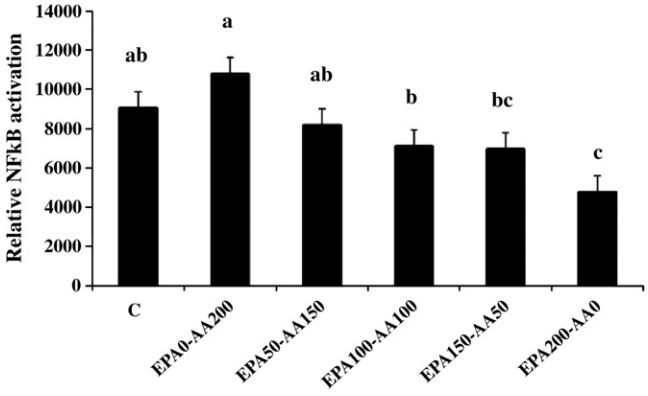

Fig. 4. EPA counteracts AA effects on NF-κB activity. 3T3-L1 cells stably transfected with NF-κB responsive reporter (NF-κB-Luc) were differentiated and treated with different AA and EPA combinations (numbers indicate the concentrations in μM), and C indicates the control with no treatment but with vehicle (DMSO). Cells were then harvested, and the NF-κB activity was assayed by luciferase reporter assay. Results represent the mean ±S.E.M. Values labeled with different letters are significantly different ($P<.05$). Values with the same letters do not differ significantly.

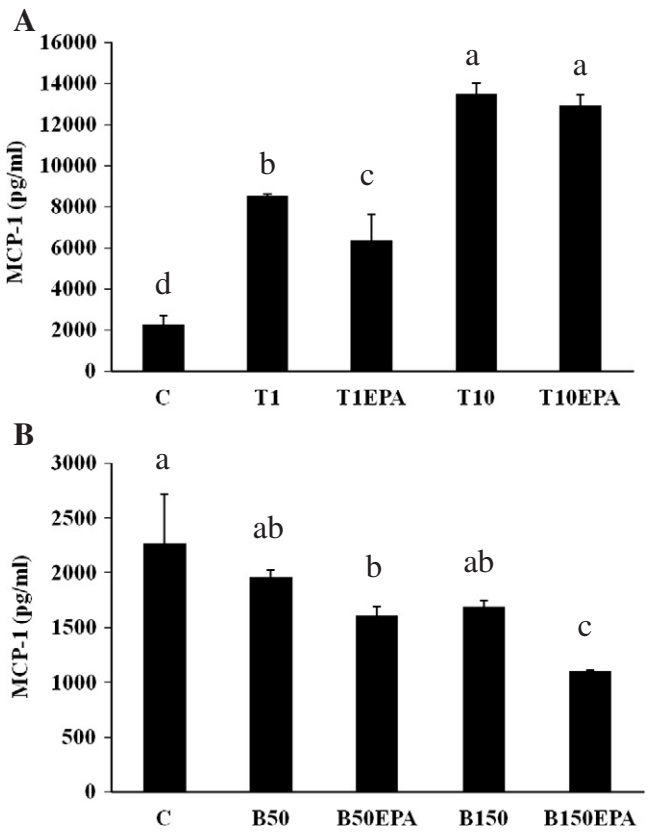

Fig. 5. Pharmacological manipulation of NF-κB activation alters adipokine secretion. 3T3-L1 cells stably transfected with NF-κB responsive reporter (NF-κB-Luc) were differentiated. EPA was co-treated with either an NF-κB activator, TNF-α (1 and 10 nM), or an NF-κB inhibitor, BAY 11-7085 (50 and 150 mM). Conditioned media were collected 48 h following these treatments, and secretion of the studied adipokines was quantified as detailed in the Methods section. Numerical values with TNF-α and BAY 11-7085 are the concentrations in nM and mM, respectively, and C indicates the control with no treatment but with vehicle (DMSO). Data shown represent triplicates for each treatment, each of which was duplicated in the ELISA assay. Results represent the mean ±S.E.M. Values labeled with different letters are significantly different ($P<.05$). Values with the same letters do not differ significantly.

7085 treatment alone (data not shown). Moreover, co-treatment of EPA and BAY 11-7085 further reduced the secretion of MCP-1 (Fig. 5B) compared to BAY 11-7085 compound alone. These results indicate that manipulation of NF-κB activity significantly alters adipokine secretion and that this effect of NF-κB is attenuated by EPA.

4. Discussion

Obesity has reached epidemic proportions worldwide and is attributed to both genetic and environmental factors as well as gene–environment interactions [36]. Metabolic complications of obesity are at least in part linked with high fat/caloric intakes and high n-6/n-3 PUFA ratios in the diet [37–39]. Thus, approaches to prevent/treat obesity include energy restriction as well as reduction in fat intake. Studies from our laboratory and others have documented beneficial effects of energy restriction in reducing metabolic complications including liver steatosis and insulin resistance [40]. However, while caloric restriction is beneficial for weight loss, it is not able to completely normalize underlying inflammation when the diet is high in saturated fat [40]. Further, it is also well documented that different types of fatty acids differentially alter metabolic functions in health and diseases [41], with saturated fatty acids being most deleterious to health and long-chain PUFAs, especially those from the n-3 family, being most beneficial to human health. These effects have been primarily attributed to n-3 PUFA anti-inflammatory effects. Further, due to high intakes of n-6 fats in the Western diet, n-3 PUFA may be even more beneficial when consumed concomitantly with lower amounts of n-6 PUFA. This is further supported with our finding that EPA showed significant anti-inflammatory activities with greater benefits with reduced AA and increased EPA levels. Also, the reported beneficial effects are at least partially due to modulation of NF-κB, a major inflammatory transcription factor.

Adipose tissue is a dynamic and complex endocrine organ which expresses and secretes a variety of bioactive compounds including proinflammatory mediators such as Agt, IL-6 and MCP-1, all of which are elevated with obesity and exert both local and systemic inflammatory effects [42–44]. Further, we reported that primary overproduction of Agt in adipose tissue leads to increaed adiposity, adipocyte hypertrophy, inflammation and insulin resistance [12,14]. These three adipokines share several common features. Ang II is a proinflammatory adipokine, and we have previously shown that Ang II treatment increases secretion of PGE_2 from cultured human and murine adipocytes [34]. Further, overexpression of Agt in adipose tissue leads to adipose and systemic inflammation and insulin resistance [14]. Similarly, increased secretion of MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance and hepatic steatosis in obesity [24]. Moreover, increased IL-6 expression has been reported in several pathogenic conditions such as obesity-related insulin resistance and CVD conditions [45,46]. Thus, suppressing secretion of all these adipocytokines (IL-6, Agt and MCP-1) can be beneficial in limiting their inflammatory effects. An obvious dietary intervention to limit their secretion includes increasing dietary intake of n-3 PUFA and limiting intake of n-6 PUFA [47].

We demonstrate here that increasing the EPA/AA ratio exerts anti-inflammatory effects on adipocytes, as indicated by the levels of secreted proinflammatory adipokines Agt, IL-6 and MCP-1. These findings are consistent with our previous report that AA but not EPA significantly increased secretion of proinflammatory PGE_2 from adipose tissue and adipocytes and that EPA significantly attenuated AA-induced PGE_2 secretion [34]. These studies further support beneficial effects of n-3 PUFAs, beyond their established cardioprotective effects, to include effects in reducing adipose tissue inflammation, a major player in metabolic disorders.

Dietary intake or treatment with AA and EPA can significantly increase production of the proinflammatory metabolite PGE_2 and the

less inflammatory PGE_3, respectively [48]. Moreover, PGE_2 induces COX-2 which promotes inflammation and cell growth, inhibits apoptosis and enhances cell motility and adhesion compared to PGE_3 [48]. Thus, PGE_2 may account for part of the AA-induced adipokine secretion compared to control (no fatty acid treatment). Moreover, the small but significant increase in MCP-1 and IL-6 in EPA 200 μM compared to control nontreated cells may be in part due to high levels of the less inflammatory PGE_3. In addition, higher doses of these highly unsaturated fatty acids may increase lipid peroxidation and/or cell toxicity. Indeed, we found that doses above 200 μM significantly decrease cell viability. However, it is worth noting that given the differential effects of EPA vs. AA in our studies, EPA effects are likely specific and not due to oxidation of this fatty acid.

In our current study (Fig. 3), we showed that EPA supplementation to HF-fed mice significantly lowered Ang II, IL-6 and MCP-1 levels compared to the HF-fed mice. However, the adipose EPA supplementation to HF diet did not change Agt levels when compared to the HF group. Given that EPA supplementation reduced Ang II levels despite having unchanged tissue Agt levels, it is plausible that EPA inhibits either Agt secretion or the activity of enzymes that cleave Agt to form Ang II. Our *in vitro* data showing increased Agt cellular content with EPA when compared to AA treatment further support a potential role of EPA in inhibiting Agt secretion or inhibiting activity of the aforementioned enzymes. Future studies are needed to determine the exact mechanisms mediating differential regulation of intracellular vs. secreted adipocyte Agt and Ang II *in vivo* and *in vitro* in response to EPA.

Further, we have consistently found that the adipocyte size in EPA-treated mice was significantly smaller than that in the counterpart HF-fed mice (data not shown), suggesting that reduced Ang II levels may be linked to changes in adipocyte size. This is in fact consistent with animal studies showing that overexpression of Agt or Ang II receptors is associated with adipocyte hypertrophy, while mice with targeted inactivation of these genes exhibit smaller adipocyte sizes compared to wild-type or transgenic mice. However, further studies are needed to confirm this observation and investigate the direct role of these adipokines in modulating fat cell size.

NF-κB activation has been implicated in many inflammatory processes [25,26]. In this study, we tested whether adipocyte-associated Agt, IL-6 and MCP-1 secretion and their regulation by PUFAs were mediated by NF-κB. Here, we report that when NF-κB activation is suppressed, MCP-1 secretion is also significantly reduced. Further, NF-κB activation was significantly increased by AA but suppressed by EPA. As an example, here we reported that secretion of MCP-1 following NF-κB activation by TNF-α was significantly reduced by EPA.

We have previously reviewed several mechanisms which may account for n-3 PUFA-mediated regulation of adipose tissue inflammation [47]. In addition to classical mediators of PUFA effects, such as prostaglandins, and AMPK regulation, a new emerging target is the G-protein-coupled receptor 120 (GPR120), which was recently reported to function as a n-3 fatty acid receptor/sensor [49]. Since GPR120 activation inhibits TNF-α signaling in macrophages [49], it is possible that in addition to suppressing NF-κB, EPA may also down-regulate Agt, IL-6 and MCP-1 secretion via GPR120 activation. Thus, further mechanistic studies focusing on GPR120-mediated EPA effects on adipokines are warranted.

In summary, our current study demonstrates anti-inflammatory effects of EPA in adipocytes, which are at least in part mediated by NF-κB. Thus, our data suggest that adding EPA to the diet will impart not only systemic but also adipose anti-inflammatory activities. Specific examples reported here are reductions of IL-6 and MCP-1 secretion, where EPA reduced both basal secretion as well as AA-induced secretion of these adipokines. Mechanistically, our studies demonstrated that NF-κB is a key mediator of AA-induced inflammation, which can be suppressed by EPA.

Additional studies in humans are critical in order to confirm these adipose anti-inflammatory effects of EPA. Given documented multiple beneficial effects of n-3 PUFA, our studies suggest that increased consumption of n-3 PUFA (as replacement of some n-6 PUFAs, i.e., changing tissue PUFA composition/ratio) will be beneficial in terms of reducing hypertensive and inflammatory markers associated with obesity.

Acknowledgments

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