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(-)-Epicatechin prevents TNF α -induced activation of signaling cascades involved in inflammation and insulin sensitivity in 3T3-L1 adipocytes

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Abstract

Obesity is major public health concern worldwide and obese individuals exhibit a higher risk of chronic diseases such as type 2 diabetes. Inflammation plays a significant role in metabolic regulation and mounting evidence highlight the contribution of adipose tissue to systemic inflammatory state. Food extracts with a high content of (-)-epicatechin have been found to exert systemic anti-inflammatory actions, however the anti-inflammatory actions of (-)-epicatechin on adipose tissue remain to be determined. The aim of this study was to investigate the capacity of (-)-epicatechin to prevent tumor necrosis alpha (TNF α)-induced activation of cell signals involved in inflammation and insulin resistance (NF- κ B, mitogen-activated protein kinases (MAPKs), AP-1, and peroxisome proliferator activated receptor γ (PPAR γ)) in differentiated white adipocytes (3T3-L1). TNF α triggered the activation of transcription factors NF- κ B and AP-1, and MAPKs ERK1/2, JNK, and p38. (-)-Epicatechin caused a dose (0.5-10 μ M)-dependent decrease in TNF α -mediated JNK, ERK1/2, and p-38 phosphorylation, and nuclear AP-1-DNA binding. (-)-Epicatechin also inhibited TNF α -triggered activation of the NF- κ B signaling cascade, preventing TNF α -mediated p65 nuclear transport and nuclear NF- κ B-DNA binding. (-)-Epicatechin also attenuated the TNF α -mediated downregulation of PPAR γ expression and decreased nuclear DNA binding. Accordingly, (-)-epicatechin inhibited TNF α -mediated altered transcription of genes (MCP-1, interleukin-6, TNF α , resistin, and protein-tyrosine phosphatase 1B) involved in

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inflammation and insulin signaling. In conclusion, (-)-epicatechin can attenuate TNF α -mediated triggering of signaling cascades involved in inflammation and insulin resistance. These findings could be of relevance in the dietary management of obesity and metabolic syndrome.

Keywords

Adipocytes; NF- κ B; MAPK; PPAR; epicatechin; inflammation; insulin resistance

Introduction

Obesity and metabolic syndrome result from excess calorie intake and genetic predisposition, and obese individuals exhibit a higher risk of chronic diseases such as type 2 diabetes [1–3]. Mounting evidence highlight the contribution of adipose tissue to a systemic inflammatory state, which can play a significant role in metabolic regulation [4, 5]. Adiposity promotes the secretion of proinflammatory cytokines, such as tumor necrosis factor alpha (TNF α), interleukin (IL)-6, resistin, monocyte chemoattractant protein-1 (MCP-1), by both adipocytes and infiltrated inflammatory cells, including recruited macrophages. These proinflammatory cytokines contribute to the sustained adipose and systemic inflammation and insulin resistance associated with obesity [6]. A significant role of TNF α on these events was initially suggested by the findings that adipose-secreted TNF α contributed to insulin resistance in rodent models of obesity [7], and that obese mice lacking a functional TNF α are protected from dietary obesity-induced insulin resistance [8]

TNF α is a major player mediating the activation of signaling cascades in adipocytes that are central to inflammation and insulin resistance. In this regard, TNF α triggers adipocyte activation of the mitogen activated kinases (MAPKs) extracellular-signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK), and p38, and of transcription factors AP-1 and nuclear factor kappa B (NF- κ B) [9–11]. These signaling cascades are in part redox regulated, given that binding of TNF α to its receptor results in the activation of NADPH oxidase leading to an increase in oxidant production [12, 13]. Activation of these signaling pathways leads to an increased expression of pro-inflammatory cytokines, including IL-6, IL-8, IL-1 β , and MCP-1. NF- κ B also drives [14] an increased expression of protein-tyrosine phosphatase 1B (PTP1B), a negative regulator of insulin signaling, which dephosphorylates tyrosine residues of the insulin receptor (IR) and insulin receptor substrate1 (IRS1) [15]. Furthermore, TNF α down regulates the nuclear receptor transcription factor peroxisome proliferator-activator receptor gamma (PPAR γ) that plays a major role in the regulation of both adipose glucose and lipid metabolism [14].

Flavonoids are polyphenolic compounds that are widely present in human diets. Epidemiological studies have shown an inverse relationship between consumption of flavonoid-rich foods and pathologies with inflammatory components [16]. The beneficial health effects of flavonoids can be in part attributed to their capacity to regulate oxidant production and pro-inflammatory signals [17]. Recent reports support an anti-inflammatory action of select polyphenols in adipocytes in cultures. Addition of a polyphenol-rich grape powder extract to human adipocytes, inhibits TNF α -triggered activation of MAPKs and NF-

κ B, and the expression of proteins involved in inflammation and insulin resistance [18]. Similarly, high concentrations of the phenolic compounds p-coumaric acid, quercetin and resveratrol prevent TNF α -induced increase in several parameters of inflammation and oxidative stress, and of decreased insulin sensitivity in 3T3-L1 [19] and human adipocytes [9]. Oligomerized grape seed polyphenols attenuated inflammatory events in co-cultures of adipocytes and macrophages, through mechanisms attributed to their antioxidative properties [20].

(-)-Epicatechin is one of the most abundant flavonoids in human diets, being present in high concentrations in grapes, cocoa, tea, and many other fruits and vegetables. (-)-Epicatechin has a basic chemical structure of two aromatic rings (A and B) linked by an oxygenated heterocycle (C) with a hydroxyl group in position 4. Consumption or supplementation with (-)-epicatechin or (-)-epicatechin-containing foods in humans and experimental animals are associated with the improvement of parameters related to cardiovascular disease, which pathology involves a significant proinflammatory component [21, 22].

In vitro, (-)-epicatechin modulates the production of reactive oxygen species, activation of NF- κ B, and production of cytokines in Jurkat T cells and Hodgkin's lymphoma cells [23, 24]. However, the capacity of (-)-epicatechin to attenuate inflammation in adipocytes has not been yet characterized. This study investigated in 3T3-L1 adipocytes, the capacity of (-)-epicatechin to inhibit TNF α -triggered deregulation of signaling pathways (MAPKs ERK1/2, JNK and p38, and transcription factors NF- κ B, AP-1, and PPAR γ) that regulate genes involved in inflammation and insulin resistance.

Materials and methods

Materials

3T3-L1 and Raw 264.7 cells were obtained from the American Type Culture Collection (Rockville, MA, USA). Cell culture media and reagents, and TRIzol reagent were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA). Antibodies for ERK (sc-93), p-Erk (sc-7383), heterogeneous nuclear ribonucleoprotein A1 (sc-32301), JNK (sc-572), p-JNK (sc-6254), PPAR γ (7273), MCP-1 (1785), TNF α (sc-1351); and the oligonucleotide containing the consensus sequence for PPAR were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The antibody for p-p38 (9211) was obtained from Cell Signaling Technology (Danvers, MA, USA). The antibody for PTP1B was from Abcam Inc. (Cambridge, MA). PVDF membranes were obtained from BIO-RAD (Hercules, CA, USA) and Chroma Spin-10 columns were from Clontech (Palo Alto, CA, USA). The ECL Western blotting system was from GE Healthcare (Piscataway, NJ, USA). The oligonucleotides containing the consensus sequence for NF- κ B, and AP-1, the reagents for the electrophoretic mobility shift assays (EMSA), and cDNA reverse transcriptase were obtained from Promega (Madison, WI, USA). (-)-Epicatechin, TNF α , lipopolysaccharide (LPS), and all other reagents were from the highest quality available and were purchased from Sigma (St. Louis, MO, USA).

Cell culture and incubations

3T3-L1 preadipocytes were maintained in DMEM containing 25 mM glucose, 10% (v/v) new born calf serum, 50 U/ml penicillin, and 50 µg/ml streptomycin. To induce cell differentiation, 3T3-L1 pre-adipocytes were grown to confluence in culture medium containing 10% (v/v) fetal bovine serum. Confluent cells were then switched to differentiation medium (DIFM) containing 20% (v/v) fetal bovine serum, 20 nM insulin and 1 nM triiodothyronine for 48 h. Adipocyte differentiation was induced by treating cells for 48 h in DIFM further supplemented with 0.5 µM dexamethasone, 0.5 mM isobutylmethylxanthine, and 0.125 mM indomethacin (induction media). After induction, cells were returned to DIFM medium, exhibiting at day 12 a fully differentiated phenotype with massive accumulation of multilocular and unilocular fat droplets. 3T3-L1 adipocytes were treated with 0.5–10 µM (-)-epicatechin during 4 h, and subsequently treated with TNFα (20 ng/ml) for 15 min–24 h depending on the determination.

RAW 264.7 macrophages were cultured in DMEM medium supplemented with 10% (v/v) fetal bovine serum. Cells were pre-incubated for 4 h in the absence or the presence of 1 and 10 µM (-)-epicatechin, and subsequently for 24 h without or with 0.5 µg/ml lipopolysaccharide (LPS). TNFα concentration in the cell culture media was determined by ELISA using a commercial kit (BD Biosciences, San Diego, CA) according to the manufacturer's protocol.

Western blot analysis

To prepare total extracts, cells were rinsed with PBS, scraped and centrifuged. The pellet was rinsed with PBS, and resuspended in 200 µL of 50 mM HEPES (pH 7.4), 125 mM KCl contained containing protease inhibitors and 2% (v/v) Igepal. The final concentration of the inhibitors was 0.5 mmol/L PMSF, 1 mg/L leupeptin, 1 mg/L pepstatin, 1.5 mg/L aprotinin, 2 mg/L pestatin and 0.4 mM sodium pervanadate. Samples were exposed to one cycle of freezing and thawing, incubated at 4° C for 30 min and centrifuged at 15,000 x g for 30 min. The supernatant was decanted and protein concentration was measured (Bradford, 1976). Aliquots of total cell lysates containing 25–40 µg protein were denatured with Laemmli buffer, separated by reducing 10–12.5% (w/v) polyacrylamide gel electrophoresis, and electroblotted to PVDF membranes. Membranes were blotted for 2 h in 5% (w/v) non-fat milk, and subsequently incubated in the presence of corresponding primary antibodies (1:1,000 dilution for all the antibodies except for TNFα (1:500) and PTP-1B (1:5,000)) overnight at 4°C. After incubation for 90 min at room temperature in the presence of the secondary antibody (HRP conjugated) (1:10,000 dilution) the conjugates were visualized and quantified by chemiluminescence detection in a Phosphoimager 840 (Amersham Pharmacia Biotech. Inc., Piscataway, NJ).

Electrophoretic mobility shift assay (EMSA)

Nuclear fractions were isolated as previously described [25, 26] with minor modifications [27]. For the EMSA, the oligonucleotides containing the consensus sequences for NF-κB, AP-1 and PPARγ were end-labelled with [γ -³²P] ATP using T4 polynucleotide kinase, and purified using Chroma Spin-10 columns. Samples were incubated with the labelled oligonucleotide (20,000–30,000 cpm) for 20 min at room temperature in 1X binding buffer

[5X binding buffer: 50 mM Tris-HCl buffer, pH 7.5, containing 20% (v/v) glycerol, 5 mM MgCl₂, 2.5 mM EDTA, 2.5 mM DTT, 250 mM NaCl, and 0.25 mg/ml poly(dI-dC)]. The products were separated by electrophoresis in a 6% (w/v) non-denaturing polyacrilamide gel using 0.5 X TBE (Tris/borate 45 mM, EDTA 1mM) as the running buffer. The gels were dried and the radioactivity quantitated in a Phosphoimager 840.

RNA isolation and real-time PCR

RNA was extracted from cells using TRIzol reagent. cDNA was generated using high-capacity cDNA reverse transcriptase. IL-6, MCP-1, resistin and TNF α mRNA levels were assessed by quantitative real-time PCR (iCycler, BioRad) using appropriate primers (Table 1) and normalized to β -actin mRNA content.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) using Statview 5.0 (SAS Institute Inc., Cary, NC, USA). Fisher least significance difference test was used to examine differences between group means. A p value < 0.05 was considered statistically significant. Data are shown as mean \pm SEM.

Results

(-)-Epicatechin decreased LPS-induced release of TNF α in RAW 264.7 macrophages

Macrophage infiltration is a key event in sustained adipose tissue inflammation. Thus, the effects of (-)-epicatechin on LPS-induced production of TNF α in RAW 264.7 mouse macrophage cells were initially evaluated. Incubation of RAW 264.7 cells with 0.5 μ g/ml LPS caused a 65% increase in TNF α secretion. Notably, simultaneous incubation of cells with LPS and 1 μ M (-)-epicatechin had no effect, while with 10 μ M (-)-epicatechin the increased release of TNF α to the incubation media triggered by LPS was completely prevented (Fig. 1).

(-)-Epicatechin prevents TNF α -induced NF- κ B activation in 3T3-L1 adipocytes

NF- κ B is at the core of chronic inflammation. Thus, we investigated the effects of (-)-epicatechin on TNF α (20 ng/ml)-induced NF- κ B activation measuring NF- κ B-DNA binding in nuclear fractions by EMSA. Adipocytes incubated for 2 h in the presence of TNF α showed a 61% increase in NF- κ B-DNA binding compared with controls (Fig. 2A). When cells were incubated with (-)-epicatechin (0.5–10 μ M) prior and during the incubation with TNF α , a dose-dependent inhibition in NF- κ B-DNA binding was observed. The inhibition was complete at 1 μ M (-)-epicatechin. To evaluate if the effects of (-)-epicatechin were due to (-)-epicatechin interactions with NF- κ B proteins, or to upstream events, the nuclear content of the NF- κ B protein p65 (RelA) was evaluated by Western blot. TNF α caused a 56% increase in p65 nuclear content, and incubation with (-)-epicatechin (0.5–10 μ M) prevented this increase (Fig. 2B).

(-)-Epicatechin prevents TNF α -induced MAPKs and AP-1 activation in 3T3-L1 adipocytes

Activation of the MAPKs occurs downstream the binding of TNF α to its receptor. In 3T3-L1 adipocytes, TNF α (20 ng/ml) caused a 12- and 2-fold increase in JNK1/2 (Thr 183, Tyr 185) and ERK1/2 (Tyr 204) phosphorylation, respectively, and a 60% increase in p38 (Thr180/Tyr182) phosphorylation after 15 min incubation (Fig. 3A,B). Preincubation of cells for 4 h in the presence of 0.5–10 μ M (-)-epicatechin caused a dose-dependent inhibition of TNF α -induced MAPKs phosphorylation (Fig. 3A,B). At 1 μ M (-)-epicatechin, the inhibition was complete for ERK1/2 and p38 phosphorylation, and partial (33%) for JNK1/2.

Activation of the transcription factor AP-1, a downstream MAPKs target, was subsequently investigated. Incubation of 3T3-L1 adipocytes in the presence of 20 ng/ml TNF α caused a 52% increase in AP-1-DNA binding (Fig. 3C). Preincubation with 1 and 10 μ M (-)-epicatechin prevented this increase.

(-)-Epicatechin prevents TNF α -induced PPAR γ downregulation in 3T3-L1 adipocytes

TNF α downregulates PPAR γ , which is a major modulator of adipogenesis. In 3T3-L1 adipocytes, and after 24 h incubation with TNF α , a 51% decrease in PPAR γ protein levels was observed in total cell lysates. (-)-Epicatechin prevented this decrease in a dose (0.5-10 μ M)-dependent manner (Fig. 4A). Consistent with these findings, the incubation with TNF α for 2 h caused a 37% decrease in PPAR γ -DNA binding in nuclear fractions compared with control cells (Fig. 4B). (-)-Epicatechin caused a dose-dependent prevention of this decrease that reached a 50% at 1 μ M (-)-epicatechin.

(-)-Epicatechin prevents TNF α -induced transcriptional activity involved in inflammation and insulin resistance

The capacity of (-)-epicatechin to modulate the expression of genes regulated by NF- κ B, MAPKs, and AP-1 was next investigated. Incubation of 3T3-L1 adipocytes for 24 h in the presence of TNF α , led to a marked increase in the mRNA levels of the cytokines IL-6, MCP-1, and TNF α , as assessed by RT-PCR (Fig. 5A). At all the concentrations tested (0.5-10 μ M), simultaneous incubation with (-)-epicatechin, prevented those increases. Similarly, TNF α caused a 54% increase in the mRNA level of the adipocytokine resistin, and (-)-epicatechin (0.5-10 μ M) prevented this increase (Fig. 5A).

Similarly to that observed for the mRNA levels, the incubation of 3T3-L1 adipocytes with TNF α for 24 h caused a 2.2-fold increase in MCP-1 and TNF α protein levels as evaluated by Western blot (Fig. 5B).. At all the tested concentrations, (-)-epicatechin prevented these increases. TNF α also led to a significant increase in PTP1B protein levels, which was prevented by simultaneous incubation with 0.5–10 μ M (-)-epicatechin (Fig. 5B).

Discussion

Chronic inflammation is one of the major contributors to white adipose tissue insulin resistance in obesity. This is due to the associated production of pro-inflammatory cytokines (TNF α , IL-6, IL-1 β) [28]. These pro-inflammatory cytokines drive the recruitment and

activation of macrophages and other immune cells that completes a cycle of inflammation and impaired metabolic function [6]. The current work demonstrates that, at concentrations compatible with those found in human tissues (high nanomolar-low micromolar range) after dietary consumption [29-32], (-)-epicatechin inhibited TNF α -induced deregulation of adipocyte cell signals (NF- κ B, MAPKs, AP-1, PPAR γ) and the downstream upregulation of genes involved in inflammation and insulin resistance.

(-)-Epicatechin not only showed a capacity to protect adipocytes from TNF α deleterious effects, but was also effective inhibiting LPS-induced TNF α release by RAW 264.7 macrophages. At higher (-)-epicatechin and catechin concentrations, a similar finding of TNF α release prevention was described in RAW 264.7 macrophages either unstimulated or stimulated with interferon gamma [33]. Thus, the capacity of (-)-epicatechin to prevent obesity-related inflammation could be due to its actions at both, the infiltrating macrophages and adipocytes.

TNF α is a major player in adipose tissue chronic inflammation triggering the activation/inhibition of signaling cascades that perpetuate the inflammatory scenario, cause insulin resistance and hyperlipemia [10, 28]. In this regard, TNF α acts activating MAPKs, and transcription factors AP-1 and NF- κ B [10], and downregulating PPAR γ . MAPKs, NF- κ B and AP-1 activation can act promoting adipocyte inflammation and insulin resistance by: i) antagonizing PPAR γ [10], ii) inducing the expression of pro-inflammatory genes and signaling proteins [10], iii) antagonizing the insulin signaling through a direct modulation of IRS-1/2 expression and phosphorylation [10, 34, 35] and by increasing the expression of PTP1B (NF- κ B) [36], and iv) modulating the oxidative stress condition associated with inflammation. Even at low concentrations (0.5–1 μ M), (-)-epicatechin inhibited partially or totally TNF α -induced MAPKs, NF- κ B and AP-1 activation. Accordingly, TNF- α -dependent transcription of pro-inflammatory genes (MCP-1, IL-6, TNF α , resistin) was inhibited by (-)-epicatechin. PTP1B expression was upregulated by TNF α , as previously described in cultured cells and in mice [36]. The expression of this protein, central to the modulation of insulin signaling, is regulated by NF- κ B [36]. Significantly, (-)-epicatechin inhibited TNF α -induced PTP1B expression at all concentrations tested. (-)-Epicatechin also prevented TNF α -mediated increase of resistin, a proinflammatory adipocytokine which expression is in part regulated by ERK1/2 and NF- κ B [37]. It has been proposed that resistin links adiposity and diabetes [38], being found elevated in conditions of obesity and insulin resistance

PPAR γ binds different ligands (e.g. fatty acids) and is the central modulator of adipogenesis, improving adipocyte insulin sensitivity, increasing adipocyte lipid uptake, and regulating the expression of adipokines (upregulation of adiponectin, downregulation of TNF α , MCP-1, resistin) (reviewed in [14]). At the systemic level, PPAR γ -induced changes in adipokines cause a decrease in hepatic lipid accumulation and glucose output, increasing fatty acid oxidation. Importantly, PPAR γ also changes macrophages from M1 into an anti-inflammatory M2 phenotype, and decreases macrophage adipose tissue infiltration [28]. (-)-Epicatechin acted decreasing TNF α -mediated downregulation of PPAR γ in 3T3-L1 adipocytes. This positive effect of (-)-epicatechin on PPAR γ could contribute to improve the loss of insulin sensitivity associated with adipose tissue inflammation.

Flavonoids have been previously shown to have beneficial effects in adipocytes. Quercetin reduced visceral adipose inflammation in obese Zucker rats [39]. In vitro, quercetin (10-60 μ M) inhibited TNF α -induced MAPK, NF- κ B and AP-1 activation and PPAR γ inhibition, improving inflammatory and insulin resistance parameters in primary cultures of human adipocytes [9]. Similar effects were observed for a grape extract containing catechins, although the complex nature of the extract does not allow a direct extrapolation to a particular active component [20]. (-)-Catechin, an (-)-epicatechin isomer, activates PPAR γ and promotes adipocyte differentiation [40]. In immune (Jurkat T) cells (-)-epicatechin, (-)-catechin, and the B2 epicatechin dimer inhibits phorbol 12-myristate 13-acetate (PMA)-induced NF- κ B activation, in part through a decrease in the associated increased cell oxidant production [23]. Furthermore, (-)-epicatechin, (-)-catechin, and dimers B1 and B2 can directly interact with NF- κ B proteins preventing its binding to κ B DNA sites [23, 41]. Both in PMA-stimulated Jurkat T cells and in Hodgkin's lymphoma cells, (-)-epicatechin inhibited NF- κ B-regulated cytokine production [23, 24]. Accordingly, our current findings demonstrate that (-)-epicatechin also acts inhibiting TNF α -triggered NF- κ B activation, which in conjunction with its inhibitory effects on MAPKs and AP-1, leads to a decreased cytokine and PTP1B expression in 3T3-L1 adipocytes.

The mechanisms of (-)-epicatechin protective actions in adipocytes challenged with TNF α cannot be addressed from the current results. It is only evident that the inhibitory actions of (-)-epicatechin on NF- κ B activation do not occur at the nucleus, but upstream, given it prevents TNF α -triggered p65 nuclear translocation. Although the concentrations of flavonoids attainable in adipose tissue would not afford a direct antioxidant mechanism of protection [42], the regulation of NADPH oxidase by (-)-epicatechin (EC) and its metabolites [43, 44], could contribute to prevent TNF α -induced and redox regulated MAPK, NF- κ B and AP-1 activation.

The obtained results demonstrate that (-)-epicatechin, at concentrations that are attainable in human tissues, prevent the deregulation of key signaling cascades (NF- κ B, MAPKs, AP-1, PPAR γ) triggered by an inflammatory stimuli (TNF α). These signaling pathways are central to the regulation, among others, of genes required for insulin sensitivity, glycogen and fatty acid synthesis, transcriptional regulation, and cytokine-mediated inflammation [10, 45]. Thus, the obtained results suggest that (-)-epicatechin-containing diets could improve obesity-associated impaired insulin sensitivity and sustained inflammation.

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Abbreviations

DIFM	differentiation medium
EMSA	electrophoretic mobility shift assay

ERK	extracellular-signal-regulated kinases
IL	interleukin
IR	insulin receptor
IRS-1	insulin receptor substrates-1
JNK	c-Jun N-terminal kinases
LPS	lipopolysaccharide
MAPKs	mitogen-activated protein kinases
MCP-1	monocyte chemoattractant protein 1
NF-κB	nuclear factor kappa B
PPARγ	peroxisome proliferator-activator receptor gamma
PTP1B	protein-tyrosine phosphatase 1B
TNFα	tumor necrosis factor alpha

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(-)-Epicatechin (EC) inhibits TNF α -triggered inflammatory response in adipocytes. MAPK, NF- κ B, and AP-1 inhibition associates with reduced pro-inflammatory gene transcription.

In parallel, EC prevents PPAR γ downregulation, central to energy metabolism.

In conclusion, EC could provide benefits against obesity-associated inflammation.

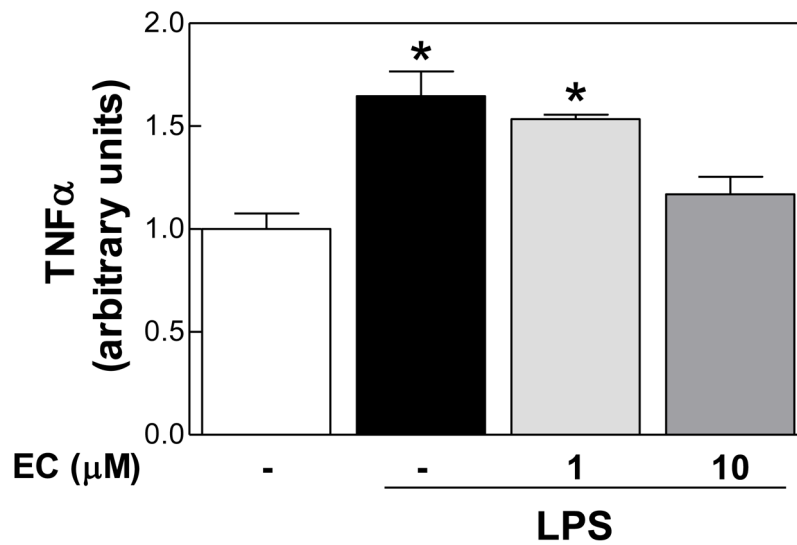


Figure 1. (-)-Epicatechin inhibits LPS-induced TNF α release in RAW 264.7 macrophages

RAW 264.7 cells were incubated without or with (-)-epicatechin (EC) (1 and 10 μ M) for 4 h, and subsequently in the absence or presence of 0.5 μ g/ml LPS for further 24 h. TNF α concentration in the media was determined by ELISA and results were referred to untreated cell values (Arbitrary unit=1). Data represent means \pm SEM of three independent experiments.

*Significantly different compared to control (no additions) ($p < 0.05$, One way ANOVA test).

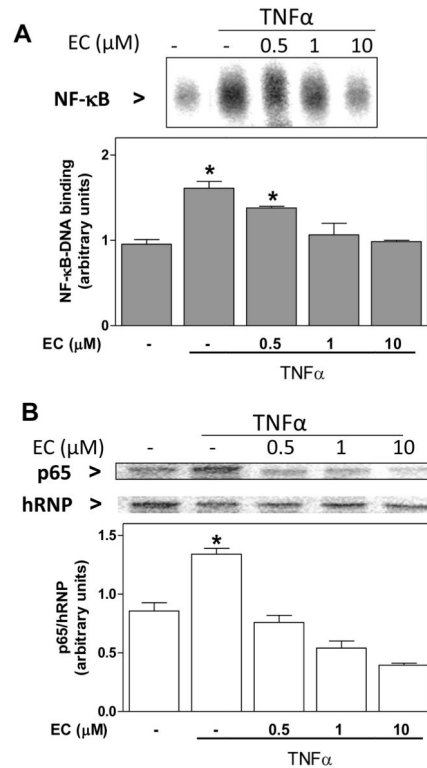


Figure 2. (-)-Epicatechin prevents TNF α -induced NF- κ B activation in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated without or with (-)-epicatechin (EC) (0.5-10 μ M) for 4 h, and subsequently in the absence or presence of 20 ng/ml TNF α for further 2 h. NF- κ B activation was evaluated measuring, A- nuclear NF- κ B-DNA binding by EMSA; and B- nuclear p65 (Rel A) levels by Western blot. Upper panels: representative images. A,B- Bands were quantified and results were referred to untreated cell values (Arbitrary unit=1). Data represent means \pm SEM of three-four independent experiments. p65 levels were referred to the nuclear content of heterogeneous nuclear ribonucleoprotein (hRNP). *Significantly different compared to all other groups ($p < 0.05$, One way ANOVA test).

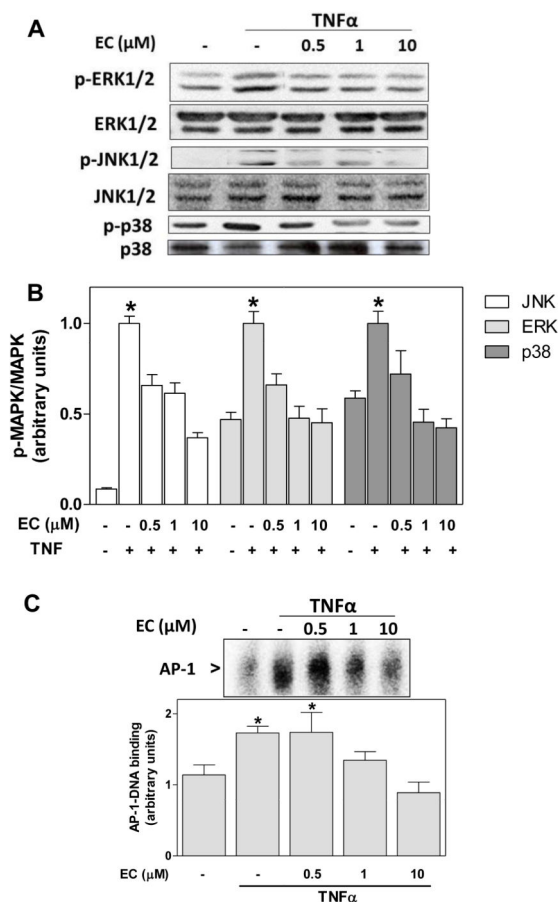


Figure 3. (-)-Epicatechin prevents TNFα-induced MAPK and AP-1 activation in 3T3-L1 adipocytes

3T3-L1 adipocytes were incubated without or with (-)-epicatechin (EC) (0.5-10 μM) for 4 h, and subsequently in the absence or presence of 20 ng/ml TNFα for further 15 min (MAPKs) or 2 h (AP-1): A- Representative images and B- quantification of Western blots for phosphorylated and non-phosphorylated JNK1/2, ERK1/2, and p38 levels in total cell extracts; C- representative image and quantification of AP-1-DNA binding in nuclear fractions as determined by EMSA. B-C, bands were quantified and results were referred to untreated cell values (Arbitrary unit=1). For Western blots (B) results were expressed as the ratio phosphorylated/non phosphorylated protein levels. Data represent means ± SEM of three to four independent experiments. *Significantly different compared to all other groups (p < 0.05, One way ANOVA test).

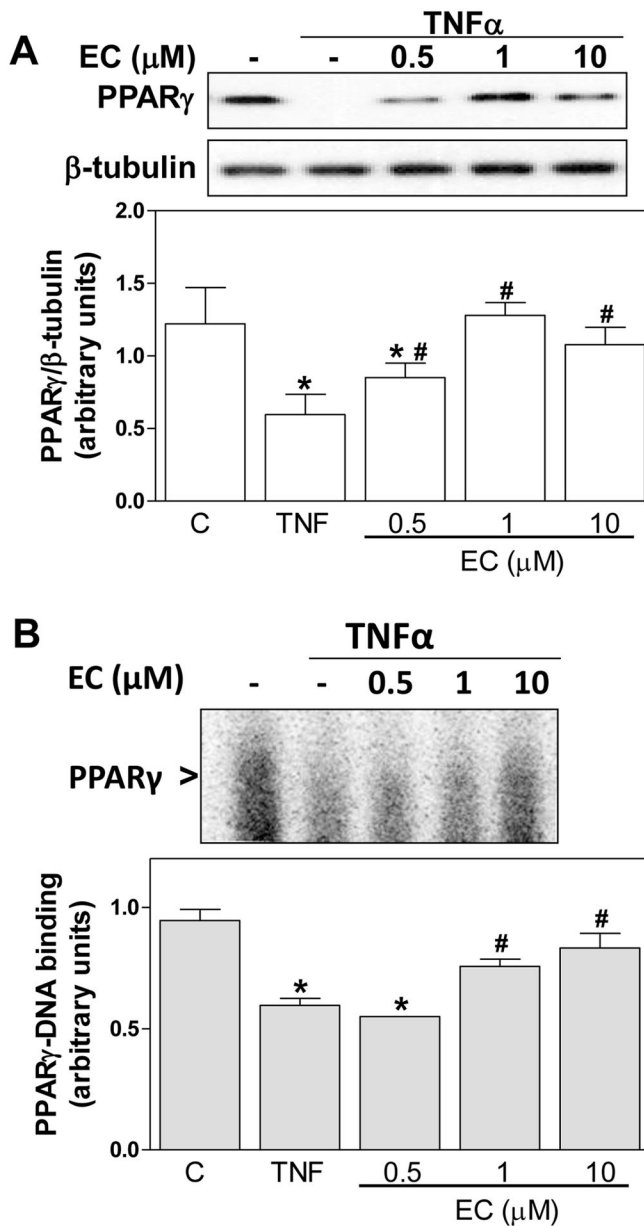


Figure 4. (-)-Epicatechin prevents TNF α -induced PPAR γ downregulation in differentiated 3T3-L1 adipocytes
 3T3-L1 adipocytes were incubated without or with (-)-epicatechin (EC) (0.5-10 μ M) for 4 h, and subsequently in the absence or presence of 20 ng/ml TNF α for further 24 h (PPAR γ protein levels) or 2 h (PPAR γ -DNA binding). A- Representative image and quantification of Western blots for PPAR γ and β -tubulin in total cell extracts, and B- Representative image and quantification of PPAR γ -DNA binding in nuclear fractions as determined by EMSA. A,B- Bands were quantified and results were referred to untreated cell values (Arbitrary unit=1). For Western blots (A), results were expressed as the ratio PPAR γ / β -tubulin protein levels. Results are shown as mean \pm SEM of 3 independent experiments. Values having different superscripts are significantly different ($p < 0.05$, one way ANOVA test).

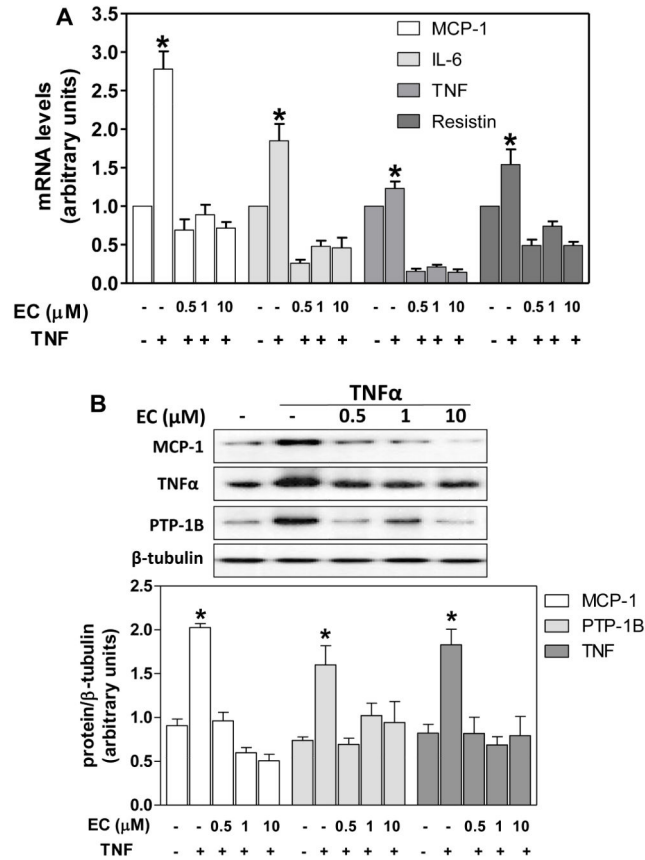


Figure 5. (-)-Epicatechin prevents TNF α -induced expression of PTP1B and of proinflammatory genes 3T3-L1 adipocytes were incubated without or with (-)-epicatechin (EC) (0.5-10 μ M) for 4 h, and subsequently in the absence or presence of 20 ng/ml TNF α for further 24 h. A- mRNA levels for MCP-1, IL-6, TNF α and resistin were measured by quantitative real-time PCR, normalized against β -actin and expressed relative to control cells (Arbitrary unit=1). B- MCP-1, PTP1B, and TNF α protein levels were measured by Western blot. Bands were quantified, results expressed as the ratio protein/ β -tubulin protein levels, and referred to untreated cell values (1). Data represent means \pm SEM of three independent experiments. *Significantly different from all other groups ($p < 0.05$, one way ANOVA test).

Table 1

Primers used for RT-PCR

Gene	5' 3' sequence	
β-Actin	<i>f</i>	CAGGGTGTGAGTTGGGAATG
	<i>r</i>	CGCACGATTTCCCTCTCAGCTG
IL-6	<i>f</i>	ACAACCACGGCCTCCCTACTT
	<i>r</i>	CAGGATTTCCCAGCGAACATGTG
MCP-1	<i>f</i>	GCCCCACTCACCTGCTGCTACT
	<i>r</i>	CCTGCTGCTGGTGATCCTCTTGT
Resistin	<i>f</i>	CAGAAGGCACAGCAGTCTTG
	<i>r</i>	GACCGGAGGACATCAGACAT
TNFα	<i>f</i>	AGCCCCAGTCTGTATCCTT
	<i>r</i>	CTCCCTTTGCAGAACTCAGG