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Inhibitory Effect of Esculetin on the Inducuble Nitric Oxide Synthase Expression in TNF-stimulated 3T3-L1 Adipocytes

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While nitric oxide (NO) produced by inducible nitric oxide synthase (iNOS) is beneficial for host survival, it is also detrimental to the host. Thus, regulation of iNOS gene expression may be an effective therapeutic strategy for the prevention of unwanted reactions at various pathologic conditions. During the screening process for the possible iNOS regulators, we observed that esculetin is a potent inhibitor of cytokine-induced iNOS expression. The treatment of 3T3-L1 adipocytes with the tumor necrosis factor-α (TNF) induced iNOS expression, leading to enhanced NO production. TNF-induced NO production was inhibited by esculetin in a dose-dependent manner. Esculetin inhibited the TNF-induced NO production at the transcriptional level through suppression of iNOS mRNA and subsequent iNOS protein expression. These results suggest esculetin, a component of natural products, as a naturally occurring, nontoxic means to attenuate iNOS expression and NO-mediated cytotoxicity.

Key Words: Esculetin, Nitric oxide, Tumor necrosis factor-α, Inducible nitric oxide synthase

INTRODUCTION

Adipose tissue is widely distributed in the body (Gray, 1989). Anatomically, adipose tissue is closely related with immune system. In mammals, nearly all lymph nodes occur always embedded in and firmly attached to adipose tissue. The adipose tissue around the lymph modes is the last depot (apart from bone marrow and the structural and cardiac depots) to be depleted in very lean wild mammals (Pond, 1996).

Acute systemic immune responses are almost invariably accompanied by anorexia and major changes in whole body lipid metabolism (Grunfeld & Feingold, 1992). The fatty acids released into the circulation are believed to be important to immune systems as an energy source, components of membrane, and substrates for the synthesis of complex lipids such as leukotrienes, prostacyclins, thromboxane, and prostaglandins (Pond, 1996).

Adipocyte itself secretes and responds vigorously to various cytokines secreted by immune cells. For example, adipose tissue appears to be an important source of tumor necrosis factor-α (TNF) and is also a target for TNF (Fruhbeck et al, 2001). The action of TNF seems to depend upon its source within body as well as its overall concentration in venous blood, strongly implicating the local interactions between adjacent tissues (Tracey & Cerami, 1992).

Nitric oxide (NO) is a free radical messenger molecule with diverse roles in vascular homeostasis, immune system and neurotransmission (Moncada et al, 1991; Jaffrey & Snyder, 1995). It is synthesized from L-arginine by NO synthase (NOS) in mammalian cells. There are at least three isotypes of NO synthase: the calcium-dependent endothelial NOS (eNOS), the neuronal type NOS (nNOS) and the relatively calcium-independent inducible NOS (iNOS). Both cNOS and iNOS have the same catalytic activity, but they differ from each other in the structure, the chromosomal localization and the regulation for gene expression (Nathan & Xie, 1994).

iNOS is implicated in host defense to a variety of inflammatory stimuli. In contrast to eNOS and nNOS, iNOS can produce large amounts of NO over the prolonged periods (Xie & Nathan, 1994). iNOS-derived NO is a double-edged sword. While NO produced by iNOS is beneficial or even critical for host survival in several infectious diseases, it is also known to be detrimental to the host. Induction of iNOS in endotoxin shock results in an enhanced formation of NO that contributes to hypotension, vascular hyporeactivity to vascular constrictors, organ injury and dysfunction (Nathan, 1997). Thus, the regulator of iNOS expression can be a useful tool to control unwanted reactions at various pathologic conditions.

During the screening process looking for possible iNOS regulators, we observed that esculetin, 6,7-dihydroxy-2H-1-benzopyran-2-one, is a potent inhibitor of cytokine-induced iNOS expression. Esculetin is a component of various natural plant products, and has been reported to exert interesting biological and biochemical activities, such as inhibition of lipoxygenases (Neichi et al, 1983; Flatman et al, 1986), anti-inflammatory effect in the Croton oil ear test

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ABBREVIATIONS: NO, nitric oxide; TNF, tumor necrosis factor-α; iNOS, inducible nitric oxide synthase; L-NAME, N-nitroso-L-arginine methyl ester.

(Tubaro et al, 1988), anti-proliferative effects on vascular smooth muscle cells (Huang et al, 1993), N-methyl-N-nitrosourea-induced mammary carcinoma (Matsunaga et al, 1998; Hecht et al, 1999), and scavenging of oxygen free radicals (Martin-Aragon et al, 1998; Lin et al, 2000). However, there has yet been no report about the effect on NO production. In the present study using 3T3-L1 adipocytes, we observed that a large amounts of NO were produced by TNF through the iNOS induction, and that esculetin inhibited the TNF-induced NO production of adipocytes.

METHODS

Culture of 3T3-L1 adipocytes

3T3-L1 cells were grown to confluence with standard medium (DMEM containing 10% fetal bovine serum, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml amphotericin B) in 75 cm^2 culture flask as described previously (Yang et al, 2002). Confluent cells were stimulated to differentiate into adipocytes by supplementing standard medium with 1 μM dexamethasone (Sigma), 10 μg/ml insulin (Sigma), and 0.5 mM isobutylmethylxanthine (Sigma) for 2 days. The medium was then replaced with standard medium containing 5 μg/ml insulin and was changed every 2 days. The cultured cells were used for experiments within 5~6 days after confluence.

Assay for nitrite

Nitrite concentration in the culture supernatant, as a reflection of NO production, was measured using a colorimetric assay (Green et al, 1982). Following a 48 h incubation in 24-well plates at 37°C in a humidified 95% air/5% CO_2 atmosphere, nitrite concentration was measured in the cell free culture supernatant. Briefly, 50 μl aliquots of the culture supernatants dispensed into 96-well microtiter plates (flat bottom) were incubated with 100 μl of a 1 : 1 mixture of 1% sulfanilamide (Sigma) in 30% acetic acid and 0.1% N-(1-naphthyl) ethylenediamine dihydrochloride (Sigma) in 60% acetic acid at room temperature. After 5 min, absorbance was measured at 540 nm using a microtiter plate reader (MR 700, Dynatech Laboratories Inc.). Concentrations were determined from a linear standard curve obtained from serial dilution of sodium nitrite (Sigma) in working medium.

Immunoblot analysis for iNOS

Cells were homogenized in 100 μl of ice cold lysis buffer (20 mM HEPES, pH 7.2, 1% Triton X-100, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 10 μg/ml aprotinin). The homogenates containing 20 μg of protein were resolved by SDS-PAGE with 10% resolving and 3% acrylamide staking gel (Laemmli, 1970), and transferred to nitrocellulose sheets (Schleicher & Schuell, Dassel, Germany) in a Western blot apparatus (Bio-Rad) run at 50 V for 2 hr. The nitrocellulose paper was blocked with 2% bovine serum albumin and then incubated for 4 hr with 1 μg/ml anti-mouse macrophage iNOS rabbit IgG (Transduction Laboratories). The binding of antibody was detected with anti-rabbit IgG conjugated to an alkaline phosphatase (Sigma). Immunoblots were developed using a BCIP/NBT solution (Pierce).

RNA isolation and RT-PCR analysis for iNOS

Total cellular RNA was prepared using Tri-zol solution (Gibco-BRL, Paisley, UK) according to the manufacturer's instructions. After the preparation of cDNA with oligo d(T)16 as a reverse transcriptase primer from the extracted RNA, amplification by PCR was performed using GeneAmp kit (Perkin Elmer, Foster City, U.S.A.) according to the manufacturer's manual. The oligonucleotide primers used for PCR are as follows: iNOS upstream 5'-CCA CAA TAG TAC AAT ACT AC-3', downstream 5'-ACG AGG TGT TCA GCG TGC TC-3'. β-actin upstream 5'-TGC CCA TCT ATG AGG GTT ACG-3', down stream 5'-TAG AAG CAT TTG CGG TGC ACG-3'. For each cDNA amplification the product of about 1 μg of total RNA was used. The reaction was cycled 30 times through 30 sec at 94°C, 45 sec at 60°C, and 45 sec at 72°C. Fifty percent of reaction mixture was analyzed by electrophoresis on 1.5% agarose gels and stained by ethidium bromide. To check the reproducibility of the results, each experiment was carried out more than three times.

Statistical analysis

Statistical analysis of the data was performed with Student's t-test. Differences with $P<0.05$ were considered statistically different.

RESULTS & DISCUSSION

The 3T3-L1 cells used for the experiment had been fully differentiated into adipocytes. Biologically produced NO is rapidly oxidized to nitrite and nitrate in aqueous solutions (Moncada et al, 1991), and incubation of the 3T3-L1 adipocytes with tumor necrosis factor-α (TNF) caused the accumulation of nitrite in the culture medium (Fig. 1). TNF-induced nitrite production was dose-dependent, with 50% of maximal nitrite accumulation occurring at a TNF

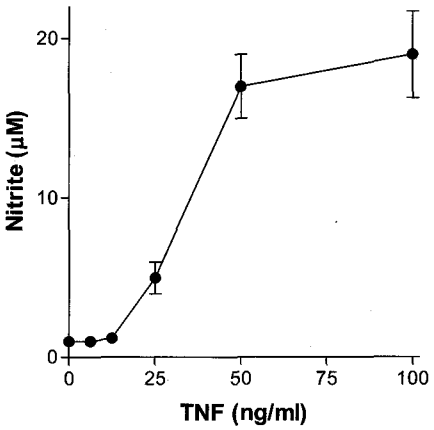

Fig. 1. Dose-dependent production of NO by TNF in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with various concentrations of TNF for 24 h. Nitrite in the medium was measured as described in Materials and Methods. Each point represents mean± S.E.M. of three independent experiments.

dose of about 28 ng/ml and reached to the plateau at the concentration of 50 ng/ml TNF. Almost no cytotoxicity was detected by MTT assay even at the concentration of 100 ng/ml TNF (data not shown). These results suggest that nitrite accumulation in the culture medium of 3T3-L1 adipocytes can be induced by TNF, most likely due to increased NOS activity.

The exact role of NO in adipocyte is so far unknown. However, there are reports that adipose tissue is a potential site of NO production in endotoxemia (Ribiere et al, 1996; Kapur et al, 1999). After lipopolysaccharide challenge, adipose tissue NOS expression increased markedly to levels comparable to that of the liver and greater than those of muscle and kidney (Kapur et al, 1999). Therefore, considering the mass and diffuse distribution, adipose tissue could significantly contribute to the overall local NO production. Adipocyte itself secretes a various adipokines such as TNF (Hotamisligil et al, 1993), leptin (Gainsford et al, 1996), adipsin (Rosen et al, 1989) or resistin (Shuldiner et al, 2001), and also responds vigorously to various cytokines secreted by immune cells (Fruhbeck et al, 2001). Thus, adipose tissue seems to represent an important participants of immune reactions, and the concerted action of TNF, NO production and other cytokines strongly support a role in immune function.

As shown in Fig. 2, stimulation of the cells with TNF (50 ng/ml) for 24 h induced high levels of nitrite (Fig. 2). However, treatment of the cells with TNF in the presence of 30 μg/ml esculetin significantly decreased nitrite production in response to TNF. The presence of esculetin diminished the cytokine-mediated nitrite formation by the 3T3-L1 adipocytes in a concentration-dependent fashion with a half maximal inhibition at 10 μg/ml. Almost complete inhibition of NO production was observed at 30 μg/ml esculetin concentration (Fig. 3). Esculetin itself did not affect the TNF-induced NO production. An iNOS inhibitor, N-nitroso-L-arginine methyl ester (L-NAME) inhibited NO production by TNF.

Lysate of 3T3-L1 adipocytes treated with TNF was subjected to immunoblot analysis, using an antibody prepared against the mouse macrophage iNOS. A major band of 130 kDa was detected in the adipocytes treated with 50 ng/ml TNF for 24 h (Fig. 4A, lane 2), and iNOS band was not detected in unstimulated adipocytes (lane 1). These results indicate that TNF-induced nitrite accumulation in the culture medium occurred through the induction of iNOS protein.

TNF-induced expression of iNOS protein was significantly inhibited by co-treatment with esculetin (Fig. 4A, lane 4). Esculetin alone did not cause the induction of iNOS protein (lane 3), however, it caused significant inhibition of NO production induced by TNF (Fig. 2). L-NAME itself did not induce the expression iNOS proteins (lane 6), and also did not affect the TNF-induced expression of iNOS protein (lane 5).

To examine whether esculetin inhibits NO production via the suppression of iNOS gene expression, the changes of

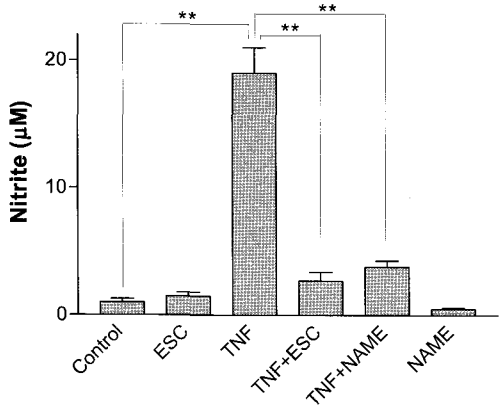

Fig. 2. Effect of esculetin on the TNF-induced production of NO in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with 50 ng/ml TNF in the presence or absence of 30 μg/ml esculetin or 1 mM L-NAME for 24 h. Nitrite in the medium was measured as described in Materials and Methods. Each point represents mean ± S.E.M. of three independent experiments. **P < 0.01.

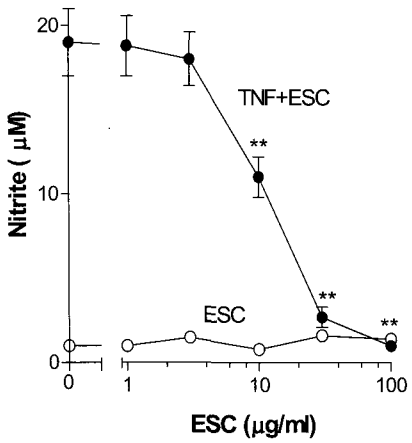

Fig. 3. Dose-dependent inhibition of the TNF-induced NO production by esculetin in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with various concentrations of esculetin in the presence or absence of 50 ng/ml TNF for 24 h. Nitrite in the medium was measured as described in Materials and Methods. Each point represents mean ± S.E.M. of three independent experiments. **P < 0.01 vs TNF only.

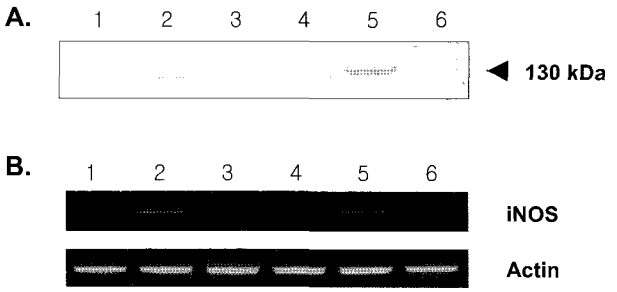

Fig. 4. Effect of esculetin on the expression of iNOS protein (A) and mRNA (B) in 3T3-L1 adipocytes. 3T3-L1 adipocytes were incubated with 50 ng/ml TNF, 30 μg/ml esculetin and/or 1 mM L-NAME for 24 h. iNOS protein and mRNA were visualized by immunoblotting with antibody for mouse macrophage iNOS (A) and by RT-PCR (B) as described in Methods, respectively. Typical result from at least three separate experiments with similar results is presented. Lane1, none; lane 2, TNF; lane 3, esculetin; lane 4, TNF and esculetin; lane 5, TNF and L-NAME; lane 6, L-NAME.

iNOS mRNA were investigated by RT-PCR (Fig. 4B). While TNF caused marked increase of iNOS mRNA (lane 2), esculetin significantly inhibited the increase of TNF-induced iNOS mRNA (lane 4). Esculetin (lane3) or L-NAME (lane 6) alone did not affect the mRNA of iNOS, and L-NAME could not inhibit the TNF-induced increase of iNOS mRNA (lane 5). These results indicate that esculetin inhibits the TNF-induced NO production at the transcriptional level through the suppression of iNOS mRNA and subsequent iNOS protein expression.

Recently, it was reported that lipoxygenase products could increase iNOS production in interleukin-1β-stimulated vascular smooth muscle cells (Hashimoto et al, 2003). As esculetin is a well-known inhibitor of lipoxygenases (Neichi et al, 1983; Flatman et al, 1986), it is highly possible that lipoxygenase products play roles in the suppressive effect of esculetin on iNOS induction. It should be mentioned, however, that esculetin has also been reported to inhibit the proliferation of vascular smooth muscle cells via a lipoxygenase-independent pathway. Esculetin inhibits the activation of p42/44 mitogen activated protein kinase (MAPK) (Pan et al, 2003), and there are several lines of evidence to suggest that p42/44 MAPK plays a role in the regulation of NF-κB activity (Hoshi et al, 2000) which is an important transcriptional regulator of iNOS expression (Nathan & Xie, 1994). In the present study, the inhibition of MAPK-mediated signaling by esculetin might have blocked the NF-κB-related cascade and subsequent iNOS expression. However, the more precise mechanism of esculetin to inhibit iNOS gene expression in adipocytes requires further study.

Excessive NO production by overexpression of iNOS gene is seen in acute and chronic diseases (Moncada et al, 1991; Xie & Nathan, 1994; Nathan, 1997). Thus, down-regulation of iNOS gene expression may be an effective therapeutic strategy for prevention of related diseases. Here, we presented evidences that esculetin is a potent suppressor of TNF-induced iNOS gene expression. These results suggest esculetin, a component of natural products, as a naturally occurring, nontoxic means to attenuate iNOS expression and NO-mediated cytotoxicity.

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