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Original article

Inhibiting insulin resistance mechanisms by DTS phytocompound: an experimental study on metabolic syndrome-prone adipocytes

Roberto Catanzaro[1], Aldo Lorenzetti[2], Flavio Allegri[3], Hariom Yadav[4], Umberto Solimene[5], Ajit K. Kumaraju[6], Emilio Minelli[5], Claudio Tomella[2], Ascanio Polimeni[2], Francesco Marotta[2]

[1] Dept. of Internal Medicine, University of Catania, Catania, Italy; [2] ReGenera Research Group for Aging-Intervention, Milano, Italy; [3] Dept of Internal Medicine, FBF Hospital, Milano, Italy; [4] National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD, USA; [5] National Cancer Institute Fredrick, MD, USA; [6] WHO-cntr for Traditional Medicine & Biotechnology, University of Milano, Italy

Abstract. The present study was designed to determine whether DTS a phytocompound endowed with antioxidant properties, could beneficially modulate nitric oxide (NO) production stimulated by lipopolysaccharide (LPS) and tumor necrosis factor-α (TNF-α) in adipocytes. Combined stimulation (CS-treatment) exerted by using 5 μg/ml of LPS together with 100 ng/ml of TNF-α significantly enhanced NO production in 3T3-L1 adipocytes. Preincubation of the adipocytes with DTS (10-30 mM) inhibited such phenomenon in a dose-dependent fashion. The production of NO was decreased by 52% at the concentration of 30mM of DTS. The decrease in NO production by DTS was associated also with a decrease in inducible nitric oxide synthase (iNOS) protein and iNOS mRNA expression. Nuclear factor-kappa B (NF-κB) was significantly enhanced by CS-treatment, while the pretreatment with 30 mM of DTS prevented the activity by 27%. IL-6 production in 3T3-L1 adipocytes was markedly increased by CS stimulus, and the enhanced secretion of IL-6 was suppressed in a dose-dependent manner by DTS. These results suggest that DTS regulates iNOS expression and NO production in adipocytes through the modulating activation of NF-κB and may have a potential clinical application within protocols designed for treating metabolic syndrome. (www.actabiomedica.it)

Key words: nuclear factor-κB, cytokines, oxidative stress, DTS

Introduction

Nitric oxide (NO), which is synthesized from the amino acid L-arginine by the enzyme NO-synthase, is intricately involved with maintaining a host of physiological processes including host defense, neuronal communication and the regulation of vasomotor tone and arterial pressure, the immune system, neurotransmission, liver physiology and others (1, 2). The primary targets for NO are metals and thiols and NO can bind to soluble gualylylcyclase and cause an increase in second messenger cGMP and mediate a number of physiological functions. Indeed, NO is a multifunctional signaling molecule and one of the most important signaling molecules in our body, and loss of NO function is one of the earliest indicators or markers of disease. Clinical studies provide evidence that insufficient NO synthesis and/or release is associated with all major cardiovascular risk factors, such as hypercholesterolemia, diabetes, hypertension, smoking and severity of atherosclerosis, and also has a profound predictive value for disease progression

including cardiovascular and Alzheimer disease. Generally speaking, NOS can be divided into two categories, i.e. a constitutive NOS, endothelial NOS (eNOS) and neuronal NOS, whose activity is dependent on intracellular calcium ion and an inducible NOS (iNOS), which is synthesized de novo in response to a number of stimuli such as cytokines, stress and radiation. At low concentrations, NO plays a role in several physiological processes but it can turn out to be toxic at high concentrations (3). Once iNOS is induced, it remains active for a certain period of time and continuously produces large amounts of NO. Peroxynitrite (ONOO-), generated by a reaction of NO with superoxide (0_2-), is a reactive nitrogen species and plays a major role in the cytotoxic process via nitration (4). Thus, iNOS-mediated NO can have a multimodal, i.e. beneficial or deleterious, effect to tissues.

In this context, one has to consider that fat is a major source of NO stimulated by leptin, an adipocyte hormone. Lipopolysaccharide activates cytokine and inducible NOS (NOS3) production in the cardiovascular system leading to CHD. As fat stores increase, leptin and NO release increases. NO could be responsible for increased CHD as obesity supervenes. 3T3-Ll cells are derived from the mouse embryonic fibroblast cell line. This cell line has the unique characteristic of accumulating large amounts of triglyceride and is accordingly used as an in vitro model of white adipocytes to study cellular mechanisms involved in obesity, metabolic syndrome and diabetes. As a matter of fact, Kapur et al. (5) have found that differentiated 3T3-Ll adipocytes induce iNOS and produce NO significantly under stimulation by LPS, tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ). Moreover, the NO produced by iNOS in adipocytes is closely linked to insulin resistance, as suggested by a recent review (6). Obesity is a multifactorial disease resulting from a combination and interaction of genetic, environmental, psychological, social, and cultural factors (7). Obesity is considered a major public health problem because it is associated with insulin resistance and diabetes. Indeed, fasting insulin is an important indicator of CHD in elderly men. Men (aged 70 to 89 years) with a high fasting insulin level have a higher prevalence of CHD and especially of myocardial infarction. Hypertension, dyslipidemia, and coronary heart diseases are other features and are characterized by increased mass of adipose tissue, which is an active endocrine and secretary organ (8, 9). Adipocytes secrete a wide range of protein signals and factors including interleukin (IL)-6, IL-1β, tumor necrosis factor (TNF-α), monocyte chemoattractant protein (MCP)-1 and adipokines such as adiponectin, leptin, and resistin. Therefore, obesity is a amplified state of inflammation (10). Thus, it has been shown that consumption of a high-fat diet promotes inflammation and the activation of NFκB (11). In a normal state, NFκB is bound with an inhibitory protein of nuclear factor-κB (IκB) in the cytoplasm. Following stimuli, such as oxidative stress and various cytokines, IκB is phosphorylated and NFκB is free to migrate into the nuclei and activate several genes such as iNOS and COX-2 (12-14).

Indeed, among other factors, oxidative stress is considered to be increased in obesity, as measured by elevated lipid and protein oxidative substances found in obese subjects (15-17).

Very recently, it has been shown that preadipocytes put in an adipogenesis-inducing cocktail manifested reactive oxygen species (ROS) via activation of the NADPH oxidase system and enahance gene epression of chemoattractant protein-1, which induces macrophage infiltration into adipose tissue and triggers inflammatory responses (18). We have recently shown that DTS, a novel nutraceutical, might exert a beneficial regulation of GSH/GSSG redox status while increasing also glutathione reductase activity and mitochondrial SOD fraction (19, 20). Therefore, we investigated whether DTS could beneficially modulate the induction of iNOS and production of NO in 3T3-Ll adipocytes,

Materials and Methods

Cell culture and reagents

3T3-Ll cells (American Type Culture Collection, Rockville, MD, USA, No. CCL 92.1) were cultured in Dulbecco's modified Eagle's medium (DMEM, Sigma Chemical Co., St. Louis, MO, supplemented with

10% (v/v) calf serum. At 2 days post-confluence, cells were induced to differentiate with DMEM supplemented with 10% (v/v) fetal bovine serum (FBS), 1 μM dexamethasone (Sigma), 0.5 mM isobutylmethylxanthine (Sigma), 1 μg/ml insulin (Novo Nordisk A/S) and ligands/inhibitors dissolved in DMSO. Cells not treated with ligands/inhibitors received similar volumes of DMSO. After 48 h, the media were replaced with DMEM supplemented with 10% FBS and 1 μg/ml insulin. The cells were subsequently re-fed every 48 h with DMEM supplemented with 10% FBS.The cells were then cultured in 6-well dishes with 2 ml of medium for nitrite and interleukin-6 (IL-6) assays, and in 100mm culture dishes with 10 ml of medium for extraction of total RNA and nuclear fractions. DTS (Kyotsu Jigyo Inst., Tokyo, Japan) solution, adjusted to pH 7.4 by 10M NaOH, was freshly prepared on the day of the treatment. The well-differentiated (>90%) 3T3-Ll adipocytes were treated for 24 hours with DTS. The cells treated with or without DTS were then exposed to the combination of 5 μg/ml of LPS (from *Escherichia coli,* Sigma) and 100 ng/ml of TNF-α (Sigma), CS-treatment, for the next 24 hours. Nω-nitroL-arginine methyl ester (L-NAME, Sigma) and aminoguanidine hydrochloride were used as iNOS inhibitors. Adipocytes cultured without these reagents for the same length of time served as control.

Measurement of nitrite production

Nitrite production was colorimetrically quantified using the Griess Assay as an index of NO production by adipocytes following exposure to inflammatory stimuli for a period of 4 h to ensure reproducibility of this assay. Nitrite standards (0–80 μM) were prepared using sodium nitrite solution in distilled H_2O and working Griess reagent was prepared using equal parts of stock Griess A (1% sulfanilamide in 5% ortho-phosphoric acid) and Griess B [1% *N*-(1-napthyl)-ethylenediamine dihydrochloride] solutions. Nitrite standards or conditioned media was added to working Griess reagent into a 96-well plate, and absorbance was measured at 540-550 nm. Nitrite concentrations were calculated from a standard curve derived from nitrite ion standard solution

Immunoblot analysis for iNOS

SDS-PAGE was performed using a 10% slab gel for iNOS as described by Laemmli (21). Forty μg of proteins from cell homogenates using CelLytic™ M (Sigma) and molecular weight marker (Nakarai Tesque, Inc., Kyoto, Japan) were loaded in each lane. After electrophoresis, the proteins were transferred onto a polyvinylidenedifluoride membrane and incubated overnight at 4°C with 1:1000 rabbit anti-mouse iNOS polyclonal antibody (Biomol Research Laboratories, Inc., PA, USA) followed by incubation with 1:1000 goat anti-rabbit horseradish peroxidase conjugated IgG, $F(ab')_2$ fragment (Sigma) for 60 min at room temperature. The peroxidase activity was evaluated by the light emitting method, ECL plus™, (Amersham Pharmacia Biotech, Buckinghamshire, England, U.K.).

Preparation of nuclear extracts

Sixty minutes after the application of CS-treatment, the cells were rinsed twice with ice-cold phosphate buffered saline with phosphates inhibitors (pH 7.4) and were scraped and submitted to centrifugation at 4°C. The pellet was then resuspended and incubated for 30 minutes in ice-cold hypotonic buffer (20 mM HEPES, 5 mM NaF, 10 μMNa_2 MoO_4, 0.1 mM EDTA, 0.5% Nonidet P-40, pH 7.5) and centrifuged at 14000 x g for 1 min at 4°C. The nuclear pellet was resuspended in ice-cold lysis buffer (20 mM HEPES pH 7.5, 0.35 M NaCl, 20% glycerol, 1% Igepal-CA630, 1mM $MgCL_2$, 0.5 mM EDTA, 0.1 mM EGTA, 5 mM DTT) containing a protease inhibitor cocktail (Gibco BRL). After 10 min of incubation at 4°C, the lysate was centrifuged for 10 minutes at 15000 x g and the supernatant was stored at -80 °C.

NF-κB activation assessment

The nuclear extracts were used for the sensitive ELISA-based kit (Active Motif North America, CA, USA) and quantify NF-κB activity according to the manufacturer's protocol as follows. The 5 μg protein of nuclear extracts were incubated for 1 hour at 25°C with oligonucleotides containing an NF-κB binding consensus which was coated to the microwells, in the presence

of competitive binding with the wild-type or mutated consensus oligonucleotides (the latter has no effect on NF- κB binding). Then, 1:1000 rabbit anti-NF*k* B p65 antibodies were incubated in each well for 1 hour at 25°C, followed by the incubation with 1:1000 peroxidase-conjugated goat anti-rabbit IgG for 1 hour at 25°C. The peroxidase activity was visualized by tetramethylbenzidine reaction and the optical density was read at 450 nm. A protein-free medium used as the blank.

Reverse-transcriptase coupled polymerase chain reaction (RT-PCR) analysis

Six hours after each treatment, the cells were rinsed twice with PBS, and total RNA was extracted using a SV Total RNA Isolation system (Promega Co, Madison, WI, USA). RNA concentrations were determined by absorbance at 260 nm. For complementary DNA (cDNA) synthesis, 2 μg of total RNA with Oligo (dT) 15 primer, RNasin (Promega), dNTPmix (Takara Bio Inc., Shiga, Japan), SuperScriptit RT (Invitrogen Co., Rockville, MD, USA) was incubated at 42°C for 50 minutes and terminated at 95°C for 15 min., Real-time PCR was performed by the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). Each PCR was carried in a total 50 μl of the reaction mixture containing 25 μl of 2XSYBR Green Master Mix (Applied Biosystems), 5 μl of sense and anti-sense primer (0.1 μM), 13 μl diethylpyrocarbonate-treated water and 2 μl of cDNA. The sequences of the oligonucleotides were as follows: iNOS, forward primer: 5'-ACGAGGTGTTCAGCGTGCTCCACG-3'. reverse primer: 5'CCACAATAGTACAATA CTACTTGG- 3', β-actin forward primer: 5'-CGTACCACTGGCAT CGTGAT-3', reverseprimer; 5'-CTGTTGGCGTACAGGTCTTTG-3'. The PCR products were visualized under a UV transilluminator by electrophoresis in a 2% agarose gel with 0.5 XTBE Buffer (45 mMTris-borate, 1 mM EDTA), followed by staining with ethidium bromide(0.1 μg/ml).

IL-6 production assay

Twenty-four hours after CS-treatment, a culture medium was collected from each sample. IL-6 concentrations in each medium were measured by using a quantitative sandwich enzyme-linked immunoassay technique (Human IL-6 immunoassay, Bioscience, USA).

Statistical analysis

All measurements were performed in duplicate and were repeated at least three times. SPSS 15.0 software was used for analysis of the data. Comparisons of data among groups were analyzed by Kruskal-Wallis test. Further comparisons between either two groups were analyzed by Nemenyi test. The values were considered to be statistically significant at $p<0.05$.

Results

Effect of DTS on the NO production in LPS and TNF-α stimulated 3T3-Ll adipocytes

Stimulation by LPS or TNF-α alone did not significantly affect the production of NO in well differentiated 3T3-Ll adipocytes (Fig. 1). However, the combined stimulation (CS-treatment) of 5 μg/ml of

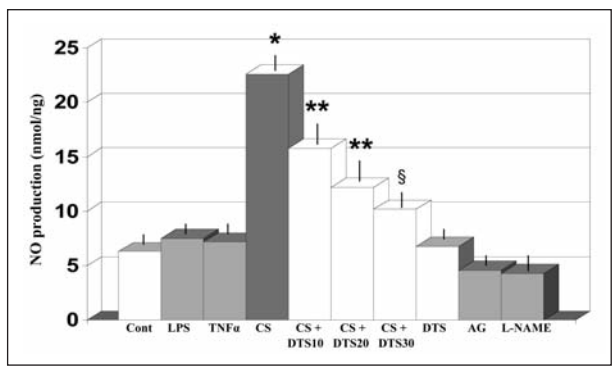

Figure 1. Effect of graded doses of DTS on the NO production in 3T3-L1 adipocytes treated with LPS and TNF-α. The nitrite concentration in the culture medium was measured 24 h after each treatment. Data were standardized by the total extractable protein, and expressed as means ± SD (n = 3-5). Cont: Control, CS: 5 μg/ml of lipopolysaccharide (LPS) + 100 ng/ml of tumor necrosis factor-alpha (TNF-α), DTS (10 - 30 mM), L-NAME: 0.5 mM of Nitro-L-arginine methyl ester. *: $p<0.01$ vs Cont; **: $p<0.05$ vs CS; §: $p<0.05$ vs other treatment groups and $p<0.01$ vs untreated CS-treated cells

LPS and 100 ng/ml of TNF-α of adipocytes significantly boosted NO production (Fig. 1). The production of NO by CS of adipocytes showed a significant dose-dependent suppression when preceeded by the preincubation with 10-30 mM of DTS. AG and L-NAME, inhibitors of iNOS, suppressed the CS -induced NO production as control. While DTS treatment alone did not affect the NO production, the CS-induced NO production was indeed reduced by 52% ($p<0.05$) at the concentration of 30 mM of DTS (Fig. 1).

Effect of DTS on the iNOS expression

Fig. 2 shows the immunoblot analysis and the RT-PCR analysis for iNOS in 3T3-Ll adipocytes. The iNOS protein band (130 kDa) and the PCR product signal (730 bp) were identified in the lanes with CS -stimulation, but not in the baseline state (control). The co-treatment with 30 mM of DTS significantly reduced the intensity of the iNOS protein and PCR product bands compared to those of CS (Fig. 2). β-actin mRNA (452 bp) as the control showed comparable signal intensity with each treatment in the adipocytes. Realtime RT-PCR analysis

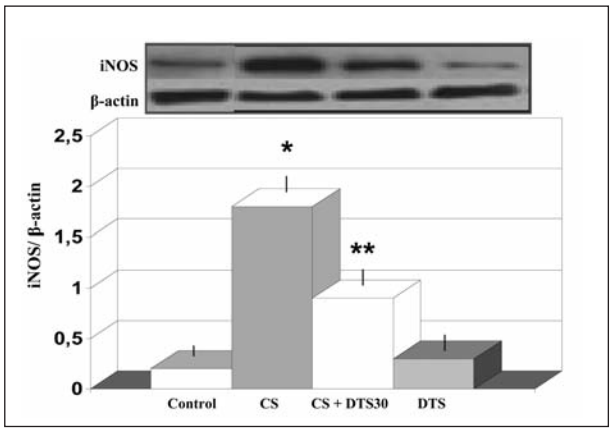

Figure 2. Effect of DTS (30mM) on the iNOS expression in CS-treated 3T3-Ll adipocytes.
The mRNA for iNOS was quantified by real time RT-PCR method in 3T3-Ll adipocytes. The PCR products were applied to agarose gel (2%) electrophoresis. Single band was depicted at 730 bp level for iNOS and at 452 bp level for β -actin. Abbreviations are the same as in fig.1. DTS: 30 mM: *: $p<0.001$ vs Cont; **: $p<0.01$ vs CS

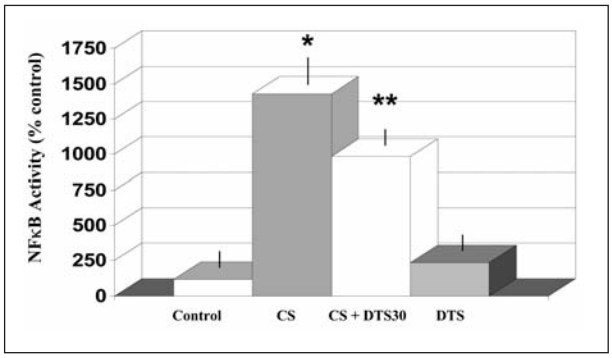

Figure 3. Effect of DTS on the CS stimulated NF- k B activation in 3T3-L1 adipocytes. The nuclear fraction of 3T3-L1 adipocytes was extracted, and assayed for free NF-κB level by ELISA. The activity was expressed as percent of the controls. Abbreviations are the same as in fig.1. DTS: 30 mM of DTS. *: $p<0.001$ vs Cont, **: $p<0.05$ vs CS

showed that DTS at 30 mM suppressed CS -induced iNOS gene expression by 42% (Fig. 2, upper panel).

Effect of DTS on the NF-κB activation

Fig. 3 shows the NF-κB activity as assayed by ELISA. NF- κB was significantly activated by the application of CS of 3T3-Ll adipocytes, while pre-treatment with 30 mM of DTS prevented the CS -mediated activation by 42% ($p<0.05$ vs lower dosages of DTS and $p<0.01$ vs untreated CS-treated cells).

Effect of DTS on the IL-6 production in CS-treated 3T3-Ll adipocytes

We investigated the effect of DTS on the production of IL-6 in CS-treated 3T3Ll adipocytes (Fig. 4). IL-6 production was markedly increased by CS -treatment and the enhanced secretion of IL-6 was suppressed in a dose-dependent manner by pre-treatment with DTS at a statistically significant level.

Discussion

Obesity and aging are two overlapping and mounting public health problems. The prevalence of obesity has been related to the increasing prevalence of metabolic syndrome, which is progressively growing

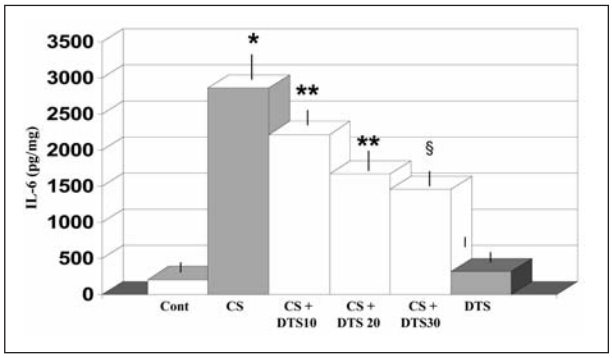

Figure 4. Effect of DTS on IL-6 production in CS-treated 3T3-Ll adipocytes. IL-6 concentration of each media was measured by ELISA as pg per mg of the total protein content of each sample. Data were expressed as means ± SD. Abbreviations are the same as in fig.1. *: p<0.001 vs Cont; **: p <0.05 vs CS; §: p<0.05 vs other treatment groups and p<0.01 vs untreated CS-treated cells. At the concentration of 30 mM DTS, the secretion of 1L-6 induced by CS stimulation was inhibited by 57%. DTS treatment alone did not affect the 1L-6 secretion

in adolescents as well as among older age groups. Indeed, insulin resistance observed with aging may be more related to adiposity than aging *per se* (22). This is because adipose tissue is an important source of TNF-α, a major pro-inflammatory factor in obesity. TNF-α contributes insulin resistance by blunting the insulin-stimulated tyrosine phosphorylation of IRS-1 (23), inhibiting glucose uptake (24), and activating NF-κB pathway (25). Although, the molecular mechanisms responsible for impaired insulin action have yet to be fully identified, rodent models demonstrate, for instance, a strong relationship between insulin resistance and an elevation in skeletal muscle inducible nitric oxide synthase (iNOS) expression (26). However, adipocytes are the main culprit and targets in such conditions and they have now been recognized as not only energy storage cells but also endocrine cells that secrete various physiologically active substances, i.e. adipocytokines (e.g., adiponectin, TNF-α, plasminogen-activator inhibitor-I) (27). In adipocytes, NF-κB signal is thus considered to have a key role in the regulation of gene expressions for adipocytokines (28, 29) and alterations of their secretions have thus been regarded as directly involved in the complications of obesity (30, 31).

In the present study, we proved that DTS inhibited the combination of LPS and TNF-α induced production of NO by inhibiting iNOS gene expression at the mRNA level via the NF- κB dependent pathway in 3T3-Ll adipocytes. In particular, pre-treatment of DTS inhibited the CS stimulated NO production dose-dependently. These data were further supported by the results on 1L-6 production. In fact IL-6 is also considered to be involved in insulin resistance and we found that DTS significantly decreased the enhanced IL-6 production in 3T3-Ll adipocytes. The signaling events induced by CS for induction of iNOS are not completely established so far in adipocytes. It has been shown that a high-fat diet does not induce insulin resistance in iNOS-knockout mice while L-NIL, a specific inhibitor of iNOS, improves hyperglycemia and insulin resistance in ob/ob mice (32). The mechanism of DTS on inhibiting the activation of NF-κB remains to be fully unfolded as yet. For instance, oxidative stress is known to be an activator of NF-κB and ROS can mediate the switching of intracellular signals (33) so that it is now established that oxidative stress contributes to the activation of NF-κB in diseases such diabetes mellitus (34) and many others. As a matter of fact, it has been shown that peroxynitrite impair insulin-stimulated glucose uptake by 3T3-Ll adipocytes (35) which represent a good model of metabolic syndrome-prone cell line. In addition, the enhanced NO production through iNOS can down-regulate the antioxidant enzyme balance and may induce oxidative stress (36). Moreover, disproportionate production of NO by iNOS may decrease insulin sensitivity in adipocytes (5). The inhibition of iNOS and pro-inflammatory cytokines by DTS in adipocytes, observed in this study, suggests that this phytocompound may indeed be significantly beneficial in the prevention of insulin resistance in vivo. Overall, we can preliminarly suggest that the observed beneficial effects might be partly explained by our previous finding that DTS might act directly on the regulation of GSH/GSSG redox status while increasing also glutathione reductase activity (19). Moreover, some saponins contained in DTS are likely to have exerted beneficial immunological effects and potent anti-inflammatory properties (37-39). Indeed, more recent experimental data highlighted the significant role of panax notoginseng saponins in rebalancing redox system while lowering inflammatory cytokines in in rats

with post-myocardial infarction (40) and acute lung injury (41). To further increase the relevance of the results, the search of the main circulating DTS metabolites are worth pursuing as well as trying lower concentrations to mimic more physiological conditions. A further limitation in this study is that we didn't perform a time-course analysis. Indeed, although prior in-house unpublished studies had shown that a plateau effect was reached by the 18h observation, a more detailed time-course sampling for analysis could provide further hints into possible different effects from potential DTS degradation molecules.

Taken altogether, these results suggest that DTS effectively regulates iNOS expression and NO production in obesity-prone adipocytes through modulating the activation of NF-κB and may have a promising potential clinical application within protocols designed for treating the emerging clinical issue of metabolic syndrome.

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Correspondence: Marotta Francesco, MD, PhD
Piazza Firenze, 12
20154 Milano, Italy
E-mail: fmarchimede@libero.it