Background: Monocytes and macrophages are critical in atherosclerosis and on stimulation secrete proinflammatory, proatherogenic cytokines such as tumor necrosis factor (TNF-α) and interleukin (IL)-1β, which have been shown to be present in atherosclerotic lesions. The aim of this study was to develop a rapid in vitro screening assay to test the antiinflammatory effects of different compounds.

Methods and Results: THP-1 cells (human monocytic cell line) were stimulated with different concentrations of lipopolysaccharide (LPS; 0 to 1000 μg/L) and for different times (4, 12, and 24 h), and the secretion of proinflammatory cytokines (IL-1, IL-6, and TNF-α) was assessed. TNF-α secretion was maximum at the lowest LPS concentration (100 μg/L) and at shortest duration of incubation (4 h). Maximum secretion of IL-1β and IL-6 was achieved at 24 h with higher doses of LPS. Treatment of THP-1 with various test compounds such as dietary supplements (α-tocopherol, N-acetylcysteine, catechin and epigallocatechin gallate) as well as pharmacologic agents (statins, peroxisome proliferator-activated receptor-γ agonists, and an angiotensin II receptor blocker) significantly inhibited LPS-stimulated TNF-α release.

Conclusions: The release of TNF-α after stimulation of THP-1 cells with LPS is a valid model system to test novel compounds for potential antiinflammatory effects.

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Atherosclerosis is a complex inflammatory process that is characterized by the presence of monocytes/macrophages and T lymphocytes in the atheroma (1). Fatty streaks, the earliest detectable lesions in atherosclerosis, contain macrophage-derived foam cells that differentiate from recruited blood monocytes. In addition, more advanced atherosclerotic lesions such as fibro-fatty plaques are the result of monocyte recruitment together with smooth muscle cell migration and proliferation. Moreover, macrophage density is greater in the shoulder region of plaques that rupture. Monocytes/Macrophages are pivotal in atherosclerosis and are present at all stages of atherosclerosis, from nascent fatty streak lesions to culmination in acute coronary syndromes (2).

Macrophages promote atherosclerosis via production of various key biomediators (3), including cytokines such as interleukin (IL)-1β,1 tumor necrosis factor-α (TNF-α), and IL-6; chemokines such as IL-8 and monocyte chemoattractant protein-1 (MCP-1); matrix metalloproteinases; and integrins (CD11b and VLA4). Although concentrations of the cytokines IL-1, IL-6, and TNF-α are increased in patients with increased risk of atherosclerosis (3–5), monocylic concentrations of these cytokines appear to be a more sensitive indicator of inflammatory status (6). Importantly, monocylic function has been reported to be altered in patients with increased cardiovascular risk including dyslipidemia, diabetes, smoking, and endstage renal disease (7–9).

Therapies that have been shown to reduce cardiovascular events appear to have antiinflammatory properties (10–17). This has been well documented with the hy-
dihydoxyethyl CoA (HMG-CoA) reductase inhibitors (statins) (10–12). Newer, specific antiinflammatory therapies are also being developed; it therefore is essential to have a simple in vitro screening assay to test their antiinflammatory effects. Because inflammation is pivotal to atherosclerosis and the monocyte/macrophage is a critical participant, we developed an in vitro model system of monocytic cells to test the antiinflammatory properties of various compounds, i.e., dietary supplements and pharmacologic agents.

**Materials and Methods**

Human monocytic leukemia THP-1 cells were obtained from the American Type Culture Collection. The cells were maintained in endotoxin-free RPMI-1640 containing 5.5 mmol/L glucose, 50 μmol/L mercaptoethanol, 100 m/L fetal bovine serum, 2 mmol/L glutamine, 1 mmol/L sodium pyruvate, and 10 mmol/L HEPES and were used for experiments between the third and fifth passages. The various cytokine ELISAs used were obtained from R&D systems. N-Acetylcysteine (NAC), vitamin E [α-tocopherol (AT)], catechin, epigallocatechin gallate (EGCG), ciglitazone, and lovastatin were obtained from Sigma Chemical Company. Candesartan was obtained from AstraZeneca as a kind gift. All of these compounds were dissolved in either dimethyl sulfoxide or ethanol. The concentration of vehicle control never exceeded 1 g/L in culture medium. A vehicle control was run in all experiments. Cell viability, as assessed by the 3-(4,5-dimethylthiazol-2-y1)-2,5, diphenyl tetrazolium bromide assay, was >95% in all experiments.

**LIPOPOLYSACCHARIDE STIMULATION OF THP-1 CELLS**

The human monocytic leukemia cell line THP-1 was chosen for this study because it is a highly differentiated monocytic cell line with phagocytic properties and has Fc receptors (C3b) as well as C3b receptors. Indeed, it is the most commonly used model to study the biology of foam cell formation because it can be easily induced to a macrophage phenotype after phorbol ester treatment (18). Furthermore, these cells have been reported to produce proinflammatory cytokines (IL-1, IL-6, and TNF) and chemokines (IL-8 and MCP-1) in response to lipopolysaccharide (LPS) stimulation (19, 20). The THP-1 cell line rather than human monocytes was used in this in vitro model system to minimize variability and to allow for high throughput.

Using the THP-1 cells, we have demonstrated that under hyperglycemic conditions superoxide anion and IL-6 release are increased, as observed in diabetic monocytes, and have elucidated the molecular mechanisms that mediate the increased superoxide anion and cytokine release from diabetic monocytes (21–23). Thus, like other investigators, we have found that the THP-1 cell line is the best in vitro model system to understand monocyte/macrophage biology as it relates to human disease.

The cells were grown in 75-mm² flasks in RPMI supplemented with fetal bovine serum until they attained 70% confluence. On reaching confluence, the cells were plated in 12-well tissue culture plates (~5 × 10⁵ cells/mL) in serum-free medium at 37 °C in 5% CO₂. The cells were challenged with different concentrations (0–1000 μg/L) of LPS for different times (4, 12, and 24 h). The supernatants were harvested after each time point and stored frozen at −20 °C until analysis. IL-1β, TNF-α, and IL-6 were quantified in all supernatants. The cells were lysed in 0.1 mol/L NaOH. The results for release of each cytokine are reported as per milligram of protein. The intra- and interassay CVs for the cytokine assays were <10%. The specific time points at which maximum cytokine stimulation was achieved were noted.

**TESTING THE ANTIINFLAMMATORY ACTIVITY OF VARIOUS COMPOUNDS**

The various compounds selected were divided into 2 categories: dietary supplements and pharmacologic agents. From our preliminary experiments, we found that TNF-α was the cytokine released at the earliest time point (4 h as opposed to 24 h for IL-1β and IL-6) and also at lowest LPS concentrations (half maximum ~50 μg/L) as discussed in Results. Therefore, for all of our future experiments testing the antiinflammatory effects of these compounds, THP-1 cells were incubated with LPS (50 μg/L) for a duration of 4 h and TNF-α concentrations were assayed in supernatants. THP-1 cells were pretreated with different concentrations of various compounds at biologically relevant concentrations. After 1 h of pretreatment with these compounds, the cells were challenged with LPS (50 μg/L) for 4 h. The supernatants were used for measurement of TNF-α. The range of TNF-α calibrators was 0–1000 ng/L.

**STATISTICAL ANALYSIS**

All experiments were repeated at least 3 times. The Student unpaired t-test was used to compute the differences, with significance set at 5%. ANOVA was used to assess dose–response effects.

**Results**

**DOSE RESPONSE FOR LPS-STIMULATED THP-1 CELLS**

THP-1 cells revealed maximum stimulation of TNF-α at an LPS concentration of 100 μg/L after a 4-h incubation (Fig. 1); however, both IL-1β and IL-6 continued to increase with maximum release after 24 h at the highest LPS concentration (~500 μg/L; Fig. 2). Whereas LPS induced the release of all 3 cytokines, TNF-α secretion was maximum at the lowest LPS concentration and shortest duration of incubation. The calculated half-maximum dose of LPS required for TNF-α secretion at 4 h was 50 μg/L. Thus, all further experiments used TNF-α as an endpoint with incubation for 4 h at an LPS concentration of 50 μg/L.
3 experiments performed in duplicate.

Cells were stimulated with different concentrations of LPS (0–1000 µg/L) for 4 (●), 12 (□), and 24 h (■). TNF-α was measured in culture supernatants as described in the Materials and Methods. Data are the mean (SD; error bars) of 3 experiments performed in duplicate.

Fig. 1. Dose response of LPS stimulation on TNF-α secretion in THP-1 cells.

EFFECT OF VARIOUS DIETARY SUPPLEMENTS ON TNF-α RELEASE

THP-1 cells were pretreated for 1 h with various compounds or vehicle control. The results for TNF-α release from THP-1 cells pretreated with various dietary supplements, AT (25, 50, and 100 µmol/L), catechin (0.25, 0.5, and 1 µmol/L), and EGCG (10–50 µmol/L) are shown in Table 1. The results revealed that AT inhibited TNF-α release significantly at all concentrations used in the study: 45%, 57%, and 62% inhibition at 25, 50, and 100 µmol/L AT, respectively. EGCG also dose-dependently inhibited TNF-α secretion (47%, 52%, and 65% inhibition at 10, 25, and 50 µmol/L EGCG, respectively). However, for NAC, TNF-α secretion was significantly inhibited only at concentrations ≥3 mmol/L (≥28% inhibition), and for catechin, TNF-α secretion was significantly inhibited only at concentrations ≥0.5 µmol/L (≥42% inhibition).

EFFECTS OF VARIOUS PHARMACOLOGIC AGENTS ON TNF-α RELEASE

The results for LPS-stimulated TNF-α release from THP-1 cells pretreated with various pharmacologic agents are shown in Table 2. All 3 drugs—lovastatin, ciglitazone, and candesartan—caused significant inhibition of TNF-α release. Lovastatin (a well-known HMG-CoA reductase inhibitor with pleiotropic effects) significantly inhibited TNF-α release at concentrations ≥1 µmol/L (43% and 49% inhibition at 1 and 5 µmol/L, respectively; P < 0.05). For ciglitazone, doses ≥2.5 µmol/L significantly inhibited TNF-α release (31% and 43% inhibition at 2.5 and 5 µmol/L, respectively; P < 0.05). Candesartan [an angiotensin receptor type I blocker (ARB)] at concentrations ≥1.0 µmol/L significantly inhibited TNF-α release (4% and 49% at 1 and 5 µmol/L, respectively; P < 0.05).

Discussion

Inflammatory processes are recognized to play a central role in the pathogenesis of atherosclerosis and its complications. The monocyte/macrophage is a crucial cell in

![Table 1. Effects of various dietary supplements on TNF-α secretion in LPS-stimulated THP1 cells.
](image)

**Table 1. Effects of various dietary supplements on TNF-α secretion in LPS-stimulated THP1 cells.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>TNF-α secretion, pg/mg of protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>82 (38)</td>
</tr>
<tr>
<td>LPS (50 µg/L)</td>
<td>4772 (875)</td>
</tr>
<tr>
<td>LPS + 25 µmol/L AT</td>
<td>2668 (465)</td>
</tr>
<tr>
<td>LPS + 50 µmol/L AT</td>
<td>2057 (622)</td>
</tr>
<tr>
<td>LPS + 100 µmol/L AT</td>
<td>1844 (331)</td>
</tr>
<tr>
<td>LPS + 0.25 µmol/L catechin</td>
<td>3243 (894)</td>
</tr>
<tr>
<td>LPS + 0.5 µmol/L catechin</td>
<td>2736 (734)</td>
</tr>
<tr>
<td>LPS + 1.0 µmol/L catechin</td>
<td>2026 (423)</td>
</tr>
<tr>
<td>LPS + 10 µmol/L EGCG</td>
<td>2515 (469)</td>
</tr>
<tr>
<td>LPS + 25 µmol/L EGCG</td>
<td>2296 (731)</td>
</tr>
<tr>
<td>LPS + 50 µmol/L EGCG</td>
<td>1655 (336)</td>
</tr>
<tr>
<td>LPS + 1 mmol/L NAC</td>
<td>3725 (923)</td>
</tr>
<tr>
<td>LPS + 3 mmol/L NAC</td>
<td>2979 (835)</td>
</tr>
<tr>
<td>LPS + 6 mmol/L NAC</td>
<td>1264 (125)</td>
</tr>
</tbody>
</table>

* The results are the mean (SD) of 3 different experiments run in duplicate.

* P < 0.001, compared with control.

** Compared with LPS stimulation alone; * P < 0.01; ** P < 0.001; *** P < 0.05.
atherosclerosis and is present in all stages of atherosclerosis. The importance of monocytes/macrophages in atherosclerosis is underscored by knock-out studies, which showed that loss of MCP-1 or its cognate receptors leads to a decrease in atherosclerosis. Monocytes/Macrophages secrete the proinflammatory cytokines IL-1, IL-6, and TNF-α. The plasma concentrations of several markers of inflammation have been found to be associated with future cardiovascular risk in a variety of clinical settings (4, 5, 9). The plasma half-life of cytokines is relatively short, and the monocyteic release of cytokines is a more sensitive and reliable indicator of inflammatory status (3).

Thus, considering the critical role of monocytes/macrophages in the inflammatory process and atherosclerosis, we developed an in vitro cell-based system using THP-1 cells after LPS stimulation to test the antiinflammatory effects of different compounds. The rationale for using LPS as the chosen agonist is based on recent data suggesting that the LPS receptors (toll-like receptor-4) are pathogenically related to atherosclerosis (24). Such a model system depicted increased secretion of IL-1, IL-6, and TNF-α after LPS stimulation. It is pertinent here to state that all of these inflammatory cytokines are reported to be increased in atherosclerotic lesions (3). In the current study, our aim was to develop a rapid, high-throughput assay as a first-line in vitro screen to provide a cost-effective strategy to test novel antiinflammatory compounds. We therefore focused only on TNF-α secretion because secretion reached maximum at the lowest concentration of LPS and at the shortest duration of incubation.

Dietary micronutrients such as AT and flavonoids have been shown to have, in addition to their antioxidant activity, antiinflammatory properties. In this system, we tested the effects of AT, catechin, EGCG, and NAC, a potent provider of sulphhydryl groups and an inhibitor of nuclear factor-kB. Catechins are flavonoid compounds that appear predominantly in green tea. Smaller amounts of catechins are also present in black tea, grapes, wine, and chocolate. The polyphenol catechins in green tea include galloacatechin, epigalloacatechin, epicatechin, and EGCG. In experimental models, catechins show a wide range of protective effects, including cardioprotective and chemoprotective properties, particularly, the tea catechin EGCG (25, 26). Furthermore, high-dose AT supplementation has been shown previously to have antiinflammatory effects (27) in diabetic patients.

In preventive cardiology, one of the major breakthroughs has been the reduction in cardiovascular events as a result of the introduction of HMG-CoA reductase inhibitors (10–12), commonly known as statins. The reduction in cardiovascular endpoints with statin use could be partly ascribed to their antiinflammatory effects (27). Numerous studies have shown that statins lower C-reactive protein (CRP) (10–12), TNF-α, IL-1, IL-6, IL-8, and MCP-1 concentrations (10). In this study, we used lovastatin, which in the AFCAPS-TEXCAPS study was shown to reduce the number of cardiovascular events and lower CRP concentrations (12). We show that in our in vitro model system, lovastatin significantly inhibited LPS-stimulated TNF-α release.

The peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists, as a class of drugs, improve insulin sensitivity and also have antiinflammatory properties (13, 14). This class of drugs has revolutionized the treatment of diabetes. They have been shown to up-regulate adiponectin. Numerous investigators have shown that PPAR-γ agonists decrease CRP (28, 29) and plasma MCP-1, TNF, serum amyloid A (29) concentrations after therapy. In addition, it has been reported that PPAR-γ has pleiotropic functions, exhibiting immunomodulatory activity in obesity as well as type 2 diabetes (13). We therefore tested the effect of ciglitazone, a PPAR-γ agonist, in our model system. Ciglitazone significantly inhibited LPS-induced TNF-α release. The reduction in TNF-α in our model system could be a mechanism explaining the reduction in insulin resistance because TNF-α impairs insulin signaling (30).

Drugs that inhibit the renin-angiotensin system, such as angiotensin-converting enzyme inhibitors and ARBs, are also known to modulate the processes of inflammation and possibly the atherosclerotic process (15, 16, 31, 32). Importantly, candesartan has also been shown to exhibit pleiotropic effects by significantly lowering plasma TNF-α concentrations in hypertensive patients (31). In our in vitro model system, candesartan (an ARB) significantly inhibited LPS-stimulated TNF-α release, confirming these in vivo findings.

Thus, the findings in this study clearly demonstrate that our in vitro model system is suitable for testing antiinflammatory effects because the dietary supplements (21, 33, 34) and pharmacologic agents (13, 16, 17, 28, 31) tested in this study have been shown to display antiinflammatory properties after chronic intake in patients/
volunteers. Importantly, TNF-α release has been implicated in cardiovascular-related diseases (30).

In conclusion, based on our results, we propose that the model system (LPS-stimulated monocytic release of TNF-α) is a rapid in vitro screening assay suitable for direct testing of compounds for antiinflammatory effects. If a compound shows antiinflammatory effects in this in vitro cell-based assay, testing should be followed by studies in relevant animal models and in human volunteers, focusing on various aspects of the inflammatory cascade (soluble cell adhesion molecules, cytokines, and chemokines, as well as downstream proteins such as serum amyloid A and CRP).

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