Xanthones from Mangosteen Inhibit Inflammation in Human Macrophages and in Human Adipocytes Exposed to Macrophage-Conditioned Media1,2

Akkarach Bumrungpert,3 Ruchaneekorn W. Kalpravidh,4 Chia-Chi Chuang,5 Angel Overman,5 Kristina Martinez,5 Arion Kennedy,5 and Michael McIntosh5*

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2Author disclosures: A. Bumrungpert, R. W. Kalpravidh, C.C. Chuang, A. Overman, K. Martinez, A. Kennedy, and M. McIntosh, no conflicts of interest.
3Department of Nutrition, Faculty of Public Health, Mahidol University, Bangkok 10400, Thailand; 4Department of Biochemistry, Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok 10700, Thailand; and 5Department of Nutrition, University of North Carolina, Greensboro, NC 27402

Abstract

Obesity-associated inflammation is characterized by recruitment of macrophages (MΦ) into white adipose tissue (WAT) and production of inflammatory cytokines, leading to the development of insulin resistance. The xanthones, α- and γ-mangostin (MG), are major bioactive compounds found in mangosteen that are reported to have antiinflammatory and antioxidant properties. Thus, we examined the efficacy of MG to prevent lipopolysaccharide (LPS)-mediated inflammation in human MΦ (differentiated U937 cells) and cross-talk with primary cultures of newly differentiated human adipocytes. We found that α- and γ-MG attenuated LPS-induced expression of inflammatory genes, including tumor necrosis factor-α, interleukin-6, and interferon-γ-inducible protein-10 in a dose-dependent manner in MΦ. We also found that α- and γ-MG attenuated LPS-activated mitogen-activated protein kinases (MAPK) and activator protein (AP)-1, but only γ-MG reduced nuclear factor-κB (NF-κB). In addition, α- and γ-MG attenuated LPS suppression of PPARγ gene expression in a dose-dependent manner. Notably, the ability of MΦ-conditioned media to cause inflammation and insulin resistance in primary cultures of human adipocytes was attenuated by pretreating MΦ with γ-MG. Taken together, these data demonstrate that MG attenuates LPS-mediated inflammation in MΦ and insulin resistance in adipocytes, possibly by preventing the activation of MAPK, NF-κB, and AP-1, which are central to inflammatory cytokine production in WAT.


Introduction

The increasing incidence of obesity and its associated morbidities has become a major public health concern worldwide [reviewed in (1)]. Obesity is associated with a chronic, low-grade inflammation characterized by macrophage (MΦ) infiltration into white adipose tissue (WAT) (2,3). MΦ contribute substantially to WAT inflammation (4). Proinflammatory factors secreted from MΦ not only induce WAT inflammation but also impair insulin signaling, leading to insulin resistance (5,6). Several studies reported that MΦ are recruited to WAT via monocyte chemoattractant protein (MCP)-1 and are the major source of proinflammatory cytokine production that contributes to systemic inflammation and insulin resistance in obese subjects (7–10). Notably, obese individuals have high circulating and tissue levels of inflammatory markers such as tumor necrosis factor-α (TNFα), interleukin (IL)-6, and MCP-1 (11,12).

Diet-induced obesity increases plasma levels of lipopolysaccharide (LPS) that contribute to inflammation and insulin resistance (13). LPS activates toll-like receptor-4 signaling in MΦs, triggering an inflammatory signaling cascade, including mitogen-activated protein kinases (MAPK), nuclear factor-κB (NF-κB), and activator protein (AP)-1, which together induce the transcription of proinflammatory cytokines such as TNFα and IL-6, contributing to inflammation and insulin resistance in WAT (13–15). Furthermore, LPS has been reported to reduce the expression and function of PPARγ, a transcription factor that transrepresses inflammatory gene transcription by antagonizing NF-κB and AP-1 activity in MΦ and adipocytes (16–17). In support of these findings, PPARγ-deficient MΦ have increased...
expression of inflammatory markers and insulin resistance (18,19). Thus, preventing the activation of MAPK, NF-κB, or AP-1 in MΦ may attenuate obesity-associated inflammation and insulin resistance.

The consumption of fruits or fruit extracts rich in phytochemicals may prevent the development of obesity-related inflammation and insulin resistance. *Garcinia mangostana* (mangosteen) is a tropical fruit native to Southeast Asia used as a traditional medicine (20). Recently, mangosteen products and chemicals may prevent the development of obesity-related inflammation and insulin resistance in WAT. However, the efficacy of MG to prevent LPS-mediated induction of inflammatory genes and adipogenesis in primary human adipocytes treated with conditioned media (CM) from activated MΦ, and its mechanism of action, are unknown.

In this study, we examined the ability of α- and γ-MG to prevent LPS-mediated induction of inflammatory genes and activation of MAPK, NF-κB, and AP-1 in human MΦ. Furthermore, we investigated the extent to which MG blocked MΦ-mediated inflammation and insulin resistance in primary human adipocytes.

**Materials and Methods**

**Materials**. All cell culture materials were purchased from Fisher Scientific. Fetal bovine serum was purchased from Hyclone. RPMI 1640 was purchased from ATCC. Tri-Reagent was purchased from Molecular Research Center. Gene-specific primers were purchased from Invitrogen, and primers for *Actb*, *Gusb*, *IκBα*, and *Gusb* were purchased from Perkin Elmer Life Science. All other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

**Preparation of MG.** α-MG (98% purity) and γ-MG (95% purity) were purified as previously described (28). Both isomers of MG were dissolved in dimethyl sulfoxide (DMSO) to make 100-mmol/L stock solutions, stored at −20°C, and diluted immediately before use.

**RNA isolation and real-time quantitative PCR.** Following treatment, cultures were harvested and total RNA was isolated using Tri-Reagent according to the manufacturer’s protocol. For real-time quantitative PCR (qPCR), 2.0 μg total RNA was converted into first-strand cDNA using Applied Biosystems High-Capacity cDNA Archive kit. qPCR was performed in an Applied Biosystems 7500 FAST Real-Time PCR system using Taqman Gene Expression assays. To account for possible variation related to cDNA input or the presence of PCR inhibitors, the endogenous reference gene GAPDH was simultaneously quantified for each sample and data were normalized accordingly.

**Immunoblotting.** Following treatment, cultures were harvested and immunoblotted as previously described (27). Briefly, total cellular protein was harvested using PBS (pH 7.5) lysis buffer containing 1% nonidet, 0.1% SDS, 0.5% SDS, 30 μL/mL aprotinin, 1 μmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L sodium orthovanadate. The samples were incubated on ice with frequent vortexing, centrifuged for 20 min at 15,000 × g, and stored at −80°C. The protein concentration was determined using the bichinichonic acid assay. The 20 μg of total cellular protein was separated by electrophoresis on 4–12% SDS-polyacrylamide gradient gels (NuPAGE mini-gel system; Invitrogen), transferred to a polyvinylidene difluoride membrane using a wet transfer module (Trans-Blot Module; Bio-Rad), and prepared for immunodetection. Following primary and secondary antibody exposure, each protein was detected using Western Lightning (PE Life Sciences) chemiluminescence substrate. Chemiluminescence was visualized following exposure of the membrane to X-ray film (X-OMAT; Eastman Kodak).

**MΦ-CM experiments in primary human adipocytes.** CM was collected from MΦ cultures pretreated for 2 h with or without 30 μmol/L γ-MG followed by treatment for 3 h with 100 μg/L LPS. LPS CM-separated from each experiment was pooled and stored at −80°C until used. For RNA isolation and qPCR experiments, primary human SV cells were seeded at 0.5 × 10^6/35-mm dish and allowed to differentiate for 6 d. On d 6, media were changed and cells were incubated in 1 mL of adipocyte media. Twenty-four hours later, 1 mL of the following treatments were added to the cultures: 1) fresh RPMI (control); 2) LPS-challenged MΦ-CM; or 3) LPS-challenged MΦ-CM pretreated with γ-MG. The duration of the MΦ-CM treatment varied depending on the outcome measured.

**Statistical analysis.** Statistical analyses were performed for data in Figures 1 and 4 by testing the main effects of MG dose (− or +) and LPS concentration on the outcome of interest.
(- or +) and their interaction (MG × LPS) using 2-way ANOVA (SPSS version 16 for Windows, SAS Institute). A 1-way ANOVA was conducted for data in Figure 5. Duncan’s tests were used to compute individual pairwise comparisons of least square means (P, 0.05). Data are expressed as means ± SEM.

Results

α- and γ-MG decrease LPS-induced inflammatory gene expression in MΦ. A preliminary time course study showed that 100 μg/L LPS treatment for 3 h robustly increased inflammatory gene expression and that 30 μmol/L γ-MG attenuated this LPS response without reducing MΦ viability (i.e. no visible difference in the number of adherent or floating cells or in cell morphology) (data not shown). Therefore, we examined the extent to which a 2-h pretreatment with 3, 10, or 30 μmol/L α- and γ-MG attenuated markers of inflammation induced by a 3-h treatment with 100 μg/L LPS. The α- and γ-MG attenuated LPS-induced increases in the expression of IL-6, TNFα, and interferon (IFN) γ-inducible protein (IP)-10 in a dose-dependent manner in human MΦ (Fig. 1A–F).

α- and γ-MG decrease LPS-mediated activation of MEK and MAPK in MΦ. Given the important role of MEK, an upstream kinase of MAPK, and MAPK in activating transcription factors that induce inflammatory gene expression, the effects of α- and γ-MG on MEK and MAPK phosphorylation in MΦ were examined. A preliminary time course study showed that 100 μg/L LPS treatment for 30 min markedly increased MAPK phosphorylation (data not shown). Thus, MΦ were pretreated for 2 h with 10 or 30 μmol/L α- and γ-MG and then stimulated for 30 min with 100 μg/L LPS. Pretreatment with γ-MG, but not α-MG, attenuated LPS-mediated IkB-α degradation (Fig. 3). Pretreatment with α- and γ-MG attenuated LPS-stimulated phosphorylation of c-Jun, a component of AP-1 and a downstream target of JNK (Fig. 3). Lastly, α- and γ-MG pretreatment attenuated LPS phosphorylation of Elk-1 and ATF-2, which are downstream targets of ERK and p38, respectively (Fig. 3).

MG decreases LPS-mediated activation of NF-κB and AP-1 in MΦ. Based on the roles that NF-κB and AP-1 (i.e. c-Jun, ATF-2, Elk-1) play in the transcriptional activation of inflammatory genes, we investigated the effects of α- and γ-MG on the activation of these proteins in human MΦ. MΦ were pretreated for 2 h with 10 or 30 μmol/L α- and γ-MG and then stimulated for 30 min with 100 μg/L LPS. Pretreatment with γ-MG, but not α-MG, attenuated LPS-mediated IkB-α degradation (Fig. 3). Pretreatment with α- and γ-MG attenuated LPS-stimulated phosphorylation of c-Jun, a component of AP-1 and a downstream target of JNK (Fig. 3). Lastly, α- and γ-MG pretreatment attenuated LPS phosphorylation of Elk-1 and ATF-2, which are downstream targets of ERK and p38, respectively (Fig. 3).
α- and γ-MG attenuate LPS-suppressed PPARγ gene expression in Mφ. Because PPARγ plays a crucial role in the transcriptional repression of inflammatory genes, we examined the effects of α- and γ-MG on PPARγ expression in human Mφ. Mφ were pretreated for 2 h with 3, 10, or 30 μmol/L α- and γ-MG and stimulated for 3 h with 100 μg/L LPS. Pretreatment with α- and γ-MG attenuated LPS suppression of PPARγ expression in a dose-dependent manner (Fig. 4A, B). In addition, pretreatment with 10 and 30 μmol/L α- and γ-MG alone increased PPARγ expression (Fig. 4A, B).

γ-MG decreases Mφ-mediated inflammation and insulin resistance in primary human adipocytes. Several studies have shown that Mφ-secreted factors cause inflammation and insulin resistance in adipocytes (5,6). Therefore, we hypothesized that MG pretreatment of Mφ would prevent Mφ-CM-mediated inflammation and insulin resistance in human adipocytes. We chose to examine γ-MG, because it appeared to be more potent than α-MG. Indeed, 30 μmol/L γ-MG pretreatment attenuated Mφ-mediated induction of inflammatory gene expression, including TNFα, IL-1β, IL-6, IL-8, and MCP-1 in human adipocytes (Fig. 5 A–E). Consistent with these data, 30 μmol/L γ-MG pretreatment blocked Mφ-CM-mediated impairment of insulin-stimulated glucose uptake in primary human adipocytes (Fig. 5F).

Discussion

In this study, we demonstrated in human Mφ that: 1) α- and γ-MG attenuated LPS-induced expression of IL-6, TNFα, and IP-10 (Fig. 1); 2) α- and γ-MG attenuated LPS activation of MEK, JNK, ERK, and p38 (Fig. 2); 3) γ-MG, but not α-MG, attenuated LPS-mediated iκB-α degradation (Fig. 3); 4) α- and γ-MG decreased LPS activation of c-Jun, Elk-1, and ATF-2 (Fig. 3); and 5) α- and γ-MG attenuated LPS suppression of PPARγ gene expression (Fig. 4). Furthermore, γ-MG prevented Mφ-CM from inducing inflammatory gene expression (Fig. 5A–E) and impairing insulin-stimulated glucose uptake in primary human adipocytes (Fig. 5F). Taken together, these new findings demonstrate that MG, especially γ-MG, inhibits LPS-mediated activation of inflammatory MAPK, transcription factors, and genes in human Mφ that cause inflammation and insulin resistance in human adipocytes.

Consistent with our findings in human Mφ, α- or γ-MG decreased inflammation in murine Mφ (24), C6 rat glioma cells (26), human adipocytes (30), and mice (24,26). Notably, the consumption of mangosteen juice blend containing MG reduced C-reactive protein levels, a biomarker of inflammation, in obese subjects compared with placebo controls (31). Notably, consuming a xanthone-rich, MG beverage containing 100 μg/L α-MG increased α-MG plasma concentrations to 0.1 μmol/L in healthy humans within 1 h (23). MG absorption increased in a dose-dependent manner and was associated with increased oxygen radical absorbance capacity. Using a simulated digestive system, Bumrungpert et al. (28) demonstrated that xanthones are digested in the gut lumen and incorporated into micelles. They are then taken up by enterocytes, incorporated into chylomicrons, transported across the basolateral membrane, and delivered to peripheral tissues. Alternatively, MG may be metabolized by Phase I-II enzymes into conjugated forms and then transported peripherally (28).

Mechanism(s) by which LPS signals to its downstream targets in Mφ have been reported. For example, LPS, which activates
MG may also suppress inflammation by inhibiting MyD88-independent IFN regulatory transcription factor (IRF)-3/IFNβ signaling in human Mφ. Support for this hypothesis comes from our data showing that MG blocks LPS-mediated expression of IP-10, which is an inflammatory chemokine regulated by MyD88-independent IRF-3/IFNβ signaling (14,36). Thus, MG may attenuate LPS-mediated induction of IP-10 expression by inhibiting MyD88-independent IRF3/IFNβ signaling in human Mφ. Future studies are needed to examine this possibility.

Mφ-secreted factors that induce inflammation and impair insulin sensitivity in adipocytes have been reported. For example, Mφ-CM increased the transcript levels of inflammation-related genes such as MCP-1, IL-6, intercellular adhesion molecule-1, and E-selectin by activating NF-κB in 3T3-L1 adipocytes (38). Similarly, our data showed that pretreatment of human Mφ-CM for 24 h and 2-[3H]deoxy-glucose uptake was measured. Data are representative of at least 3 independent experiments. Values are means ± SEM, n = 4. Means without a common letter differ, P < 0.05.

Taken together, these data demonstrate that α- or γ-MG attenuates LPS activation of MAPK, NF-κB, and AP-1 and suppression of PPARγ expression that leads to the induction of inflamma-

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tory genes in human MΦ. Moreover, the ability of MΦ-CM to cause inflammation and insulin resistance in human adipocytes is prevented by pretreating MΦ with γ-MG. However, in vivo studies supplementing obese humans or animals with MG are needed to determine the extent to which these in vitro data can be replicated in vivo.

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Literature Cited