Xanthones from Mangosteen Prevent Lipopolysaccharide-Mediated Inflammation and Insulin Resistance in Primary Cultures of Human Adipocytes\textsuperscript{1,2}

Akkarach Bumrungpert,\textsuperscript{3,7} Ruchaneekorn W. Kalpravidh,\textsuperscript{4,7} Chureeporn Chitchumroonchokchai,\textsuperscript{5} Chia-Chi Chuang,\textsuperscript{6} Tiffany West,\textsuperscript{6} Arion Kennedy,\textsuperscript{6} and Michael McIntosh\textsuperscript{6*}

\textsuperscript{3}Department of Nutrition, Mahidol University, Bangkok 10400, Thailand; \textsuperscript{4}Department of Biochemistry, Mahidol University, Bangkok 10700, Thailand; \textsuperscript{5}Department of Human Nutrition, The Ohio State University, Columbus, OH 43210; and \textsuperscript{6}Department of Nutrition, University of North Carolina, Greensboro, NC 27402

Abstract
The xanthones, α- and γ-mangostin (MG), are major bioactive compounds found in mangosteen and are reported to have antiinflammatory properties in several murine models. Given the association between obesity, chronic low-grade inflammation, and insulin resistance, we examined the effects of α- and γ-MG on markers of inflammation and insulin resistance in primary cultures of newly differentiated human adipocytes treated with lipopolysaccharide (LPS). α- and γ-MG decreased the induction by LPS of inflammatory genes, including tumor necrosis factor-α, interleukin (IL)-1β, IL-6, IL-8, monocyte chemoattractant protein-1, and Toll-like receptor-2. Moreover, α- and γ-MG attenuated LPS activation of the mitogen-activated protein kinases (MAPK) c-jun NH\textsubscript{2}-terminal kinase, extracellular signal-related kinase, and p38. α- and γ-MG also attenuated LPS activation of c-Jun and activator protein (AP)-1 activity. γ-MG was more effective than α-MG on an equimolar basis. Furthermore, α-MG but not γ-MG attenuated LPS-mediated IκB-α degradation and nuclear factor-κB (NF-κB) activity. In addition, γ-MG prevented the suppression by LPS of insulin-stimulated glucose uptake and PPAR-γ and adiponectin gene expression. Taken together, these data demonstrate that MG attenuates LPS-mediated inflammation and insulin resistance in human adipocytes, possibly by inhibiting the activation of MAPK, NF-κB, and AP-1.

Introduction
Obesity is a major public health problem with serious metabolic consequences. The incidence of obesity and its associated disorders is increasing markedly worldwide [reviewed in (1)]. Obesity is closely associated with a state of chronic, low-grade inflammation characterized by abnormal cytokine production and activation of inflammatory signaling pathways in white adipose tissue (WAT),\textsuperscript{8} resulting in the induction of several biological markers of inflammation such as tumor necrosis factor (TNF)-α, interleukin (IL)-6, and monocyte chemoattractant protein (MCP)-1 [reviewed in (2)]. These biomarkers are present at increased concentrations in individuals who are obese and insulin resistant and predict the development of type 2 diabetes mellitus or cardiovascular diseases [reviewed in (3)].

Obesity-associated inflammation in WAT is strongly linked to the development of insulin resistance. Recently, several studies reported that deletions of inflammatory cytokine genes, including TNFα, IL-6, and MCP-1, protect against the development of insulin resistance and hyperglycemia in obese mice (4–7). Moreover, disruption of Toll-like receptor (TLR) signaling in mice confers protection from obesity-induced inflammation and insulin resistance (8,9). Furthermore, activation of inflammatory signaling pathways like mitogen-activated protein kinases (MAPK) [i.e. c-jun NH\textsubscript{2}-terminal kinase (JNK), p38, extracellular signal-related kinase (ERK)] and nuclear factor-κB (NF-κB) results in insulin resistance (10,11). Conversely, the absence of JNK prevents the development of insulin resistance in murine models of obesity (12). Inhibition of NF-κB signaling using salicylates or targeted disruption of IκB kinase-β, a central coordinator of inflammatory responses through activation of NF-κB, confers protection from obesity-induced insulin resistance (13,14). In addition, activation of MAPK and NF-κB...
inhibit PPARγ activity, thereby suppressing the synthesis of adiponectin, an adipocyte-secreted protein that enhances insulin sensitivity by increasing AMP kinase activity (15,16).

Accordingly, developing strategies to reduce the activation of MAPK and NF-κB and their downstream induction of inflammatory genes in WAT could be effective in preventing obesity-associated inflammation and insulin resistance. In this regard, the evaluation of the antiinflammatory effects of various bioactive food components has gained widespread attention. In particular, the consumption of fruits or fruit extracts rich in bioactive components may prevent the development of obesity-related inflammation and insulin resistance. 

Garcinia mangostana (mangosteen) is a tropical fruit native to southeast Asia, including Thailand. Mangosteen has been used as a traditional medicine for treatment of skin infection, wounds, and diarrhea for many years (17). Recently, mangosteen products have gained popularity as functional foods and botanical dietary supplements due to their purported health benefits (18). α- and γ-mangostin (MG) are the most abundant xanthones present in mangosteen reported to have antiinflammatory activities. Previous studies demonstrated that α- and γ-MG inhibited nitric oxide and prostaglandin E2 production from lipopolysaccharide (LPS)-stimulated RAW 264.7 murine macrophages (19). Moreover, γ-MG showed antiinflammatory activity by inhibiting IkB kinase-β activity and the synthesis of cyclooxygenase-2 and prostaglandin E2 in C6 rat glioma cells (20,21).

However, the effects of α- and γ-MG on inflammation and insulin resistance in adipocytes have not been elucidated. Therefore, we examined the efficacy of α- and γ-MG to prevent LPS-mediated activation of MAPK, NF-κB, and activator protein (AP)-1 and induction of inflammatory genes as well as insulin-stimulated glucose uptake in primary cultures of newly differentiated human adipocytes.

Materials and Methods

Materials. All cell culture ware were purchased from Fisher Scientific. Fetal bovine serum was purchased from Hyclone. DNA-free was purchased from Ambion. Tri Reagent was purchased from Molecular Research Center. Gene-specific primers were purchased from Applied Biosystems. Polyclonal antibody for anti-glyceraldehyde-3-phosphate dehydrogenase was purchased from Santa Cruz Biotechnology. Anti-phospho (Thr180/Tyr182) p38 antibody was purchased from BD Biosciences Pharmingen. Anti-phospho (Thr183/Tyr185) and total JNK, anti-phospho (Thr202/Tyr204) and total ERK, total p38, and anti-phospho (Ser63) and total c-Jun antibodies were purchased from Cell Signaling Technologies. Immunoblotting buffers and pregel gels were purchased from Invitrogen. Western Lightning chemiluminescence substrate was purchased from Perkin Elmer Life Science. The Nucleofector and Dual Glo luciferase kits were obtained from Amaxa and Promega, respectively. All other reagents and chemicals was purchased from Sigma Chemical unless otherwise stated.

Culturing of human primary adipocytes. Abdominal WAT was obtained from nondiabetic females between the ages of 20 and 50 y old with a BMI <32.0 during abdominoplasty. Approval was obtained from the Institutional Review Board at the University of North Carolina at Greensboro. Tissue was digested using collagenase and stromal vascular cells were isolated and cultured as previously described (22). Cultures containing ~50% preadipocytes and ~50% adipocytes, based on visual observations, were treated between d 6 and 12 of differentiation. Each experiment was repeated at least twice at different times using a mixture of cells from 2–3 participants unless otherwise indicated.

Preparation of MG. α-MG (98%) and γ-MG (95%) were purified as previously described (23). Both isomers of MG were dissolved in dimethyl sulfoxide (DMSO) to make the concentration of 100 mmol/L as the stock solutions and stored at −20°C. Stock solutions were diluted immediately before use.

RNA isolation and real-time quantitative PCR. Primary human stromal vascular cells were seeded in 35-mm dishes at 0.5 × 10^5 per dish and differentiated for 6 d. On d 6, media was changed. Twenty-four hours later, cultures were pretreated with DMSO vehicle or 3 μmol/L α-MG or γ-MG and then treated with 10 μg/L LPS. 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 glyceraldehyde-3-phosphate dehydrogenase was simultaneously quantified for each sample and data were normalized accordingly.

Immunoblotting. We treated primary human adipocytes with MG and/or LPS as described above. Immunoblotting was conducted as previously described (22).

Transient transfections of human adipocytes. For measuring NF-κB and AP-1 activity, primary human adipocytes were transiently transfected with the NF-κB or AP-1 responsive luciferase (luc) reporter construct pNF-κB or pAP-1 luc (Strategene) using the Amaxa Nucleofector. On d 6 of differentiation, 1.2 × 10^5 cells from a 60-mm plate were trypsinized and resuspended in 100 μL of nucleofector solution (Amaxa) and mixed with 1 μg of pNF-κB or pAP-1 luc and 25 ng pRL-CMV for each sample. Electroporation was performed using the V-33 nucleofector program (Amaxa). Cells were replated in 96-well plates after 10 min of recovery in calcium-free RPMI media. After 24 h, transfected cells were treated with MG and/or LPS as described above. Firefly luc activity was measured using the Dual-Glo luciferase kit and normalized to Renilla luc activity from the cotransfected control pRL-CMV vector. All luc data are presented as a ratio of firefly luc:Renilla luc activity.

Results

MG decreases LPS-induced inflammatory gene expression. A preliminary dose-response study showed that 3 μmol/L MG most effectively decreased the mRNA levels of several candidate inflammatory genes exposed to LPS (data not shown) without reducing cell viability (i.e. no visible difference in the number of adherent or floating cells or in cell morphology). Therefore, we examined the extent to which 3 μmol/L MG attenuated markers of inflammation induced by 10 μg/L LPS in primary cultures of human adipocytes. α- and γ-MG attenuated LPS-mediated increases in the expression of TNFα, IL-1β, IL-6, IL-8, MCP-1, and TLR-2 (Fig. 1). γ-MG was more effective than α-MG on an equimolar basis. In the absence of LPS, α- and γ-MG alone did not affect inflammatory gene expression (Fig. 1). These data demonstrate that γ-MG, and to a lesser extent
α-MG and γ-MG attenuate LPS-induced inflammatory gene expression in primary cultures of human adipocytes.

**MG decreases LPS-mediated MAPK activation.** Given the important role of MAPK in activating transcription factors that induce inflammatory gene expression, we examined the effects of α- and γ-MG on MAPK phosphorylation. Pretreatment of cultures of human adipocytes with α- and γ-MG modestly attenuated LPS phosphorylation of JNK, p38, and ERK (Fig. 2). γ-MG was more effective than α-MG on an equimolar basis. In the absence of LPS, α- and γ-MG alone did not affect MAPK activation (Fig. 2). These data demonstrate that α- and γ-MG attenuate the activation by LPS of MAPK in primary cultures of human adipocytes.

**MG decreases LPS-mediated NF-κB and AP-1 activation.** Because activation of NF-κB and AP-1 play an important role in the transcriptional activation of inflammatory genes, we examined the effects of α- and γ-MG on NF-κB and AP-1 activation. Pretreatment with γ-MG, but not α-MG, attenuated IκBα degradation by LPS (Fig. 3A). Consistent with these data, γ-MG attenuated LPS-stimulated NF-κB reporter activity (Fig. 3B). Similarly, α- and γ-MG inhibited LPS-stimulated phosphorylation of c-Jun, a component of AP-1 and a downstream target of JNK (Fig. 4A). Consistent with these data, α- and γ-MG pretreatment blocked LPS-induced transcriptional activity of AP-1 (Fig. 4B). In the absence of LPS, neither α- nor γ-MG influenced NF-κB and AP-1 activation (Figs. 3 and 4). Taken together, these data demonstrate that γ-MG is more effective than α-MG in blocking LPS-mediated NF-κB and AP-1 activity in primary cultures of human adipocytes.

**MG prevents LPS-induced insulin resistance.** Inflammation in WAT is intimately linked to insulin resistance. Our previous study demonstrated that LPS causes insulin resistance in primary cultures of newly differentiated human adipocytes via the activation of MAPK and NF-κB and suppression of PPARγ and adiponectin gene expression (15). Given the antiinflammatory effects of MG, we speculated that MG would improve insulin sensitivity in cultures of human adipocytes treated with LPS. Indeed, the suppression by LPS of insulin-stimulated glucose uptake was blocked by γ-MG (Fig. 5A). Consistent with these data, γ-MG attenuated the suppression by LPS of the mRNA levels of PPARγ and adiponectin (Fig. 5B), genes needed for insulin-stimulated glucose uptake and utilization. Collectively, these data show that γ-MG prevents LPS-mediated insulin resistance in primary cultures of human adipocytes, possibly by inhibiting inflammation and the suppression of PPARγ or its target genes.

**Discussion**

In this study, we determined the extent to which α- and γ-MG prevented the induction by LPS of inflammatory adipocytokines and activation of upstream proteins that regulate their expression. These findings provide important insights into the potential use of MG as a therapeutic agent for treating inflammatory conditions associated with obesity and insulin resistance.
transcription in primary cultures of newly differentiated human adipocytes. We demonstrated that: 1) α- and γ-MG attenuated LPS-induced expression of TNFα, IL-1β, IL-6, IL-8, MCP-1, and TLR-2, an inducible gene linked to Toll-receptor signaling; 2) α- and γ-MG attenuated the activation by LPS of JNK, p-38, and ERK, MAPK linked to the activation of NF-κB and AP-1; 3) α- and γ-MG prevented the activation by LPS of c-Jun and AP-1 activity; 4) γ-MG, but not α-MG, attenuated LPS-mediated IκBα degradation and NF-κB activity; and 5) γ-MG prevented the suppression by LPS of insulin-stimulated glucose uptake and PPARγ and adiponectin gene expression. Taken together, these findings are the first to demonstrate that MG inhibits LPS-mediated activation of the MAPK p38, JNK, and ERK and the transcription factors NF-κB and AP-1 that induce inflammatory genes known to cause insulin resistance in human adipocytes.

Based on these data and our previously published data (15), we propose the following scenario by which MG reduces LPS-mediated inflammation (Fig. 1–4) and insulin resistance (Fig. 5). We speculate that MG initially attenuates LPS-induced inflammatory signaling by decreasing the expression of TLR2, an inducible gene primarily expressed in preadipocytes as opposed to adipocytes. This speculation is based on human preadipocytes being much more inflammatory than adipocytes when challenged with LPS (15) and the higher expression levels of TLR2/4 in preadipocytes than in adipocytes (15). Activation of TLR in turn, triggers activation of MAPK, NF-κB, and AP-1, thereby increasing adipocytokine production (i.e. TNFα, IL-1β, IL-6, IL-8, MCP-1) in preadipocytes. These cytokines, in turn, activate their cognate cell surface receptors on both adipocytes and preadipocytes, further augmenting cytokine production. Alternatively, MG may directly attenuate the activation by LPS/ adipocytokine of MAPK, NF-κB, and AP-1, thereby preventing their suppression of PPARγ and its target genes and insulin-stimulated glucose uptake.

Consistent with our data in human adipocytes, α- and γ-MG have been shown in vitro to reduce inflammation in RAW 264.7 murine macrophages. γ-MG had a stronger efficacy than α-MG (19). Moreover, γ-MG has been reported to inhibit LPS-mediated NF-κB activation in C6 rat glioma cells (21). Additionally, α- and γ-MG have been shown in vivo to decrease inflammation in carrageenan-induced paw edema in mice used as an acute model of inflammation (19,21). However, future animal or human studies should determine whether MG inhibits the expression of inflammatory adipokines and insulin resistance in vivo.

Mechanism(s) by which LPS signals to its downstream targets in adipocytes have been reported. For example, we previously demonstrated that TLR-2 gene expression was robustly induced by LPS, particularly in preadipocytes, which could be partially responsible for the higher inflammatory responsiveness of human preadipocytes treated with LPS (15). The induction of TLR-2 by LPS was directly linked to activation of MAPK and inflammatory genes and suppression of PPARγ activity and insulin-stimulated glucose uptake (15). In contrast, TLR-4 appeared to be constitutively expressed in human preadipocytes and adipocytes, albeit at higher levels in preadipocytes (15). Consistent with these data, LPS activates several intracellular signaling pathways, including MAPK and NF-κB. LPS has been reported to induce MAPK and NF-κB signaling through TLR in murine (pre)adipocytes (25,26).

Activation of NF-κB is considered to be the central pathway for the regulation of inflammatory cytokine expression, including TNFα, IL-1β, and IL-6. In resting cells, NF-κB proteins (e.g. p50, p65) are present in the cytoplasm tethered with IκB, an inhibitor protein subunit. After stimulation by LPS, a serine kinase cascade is activated, leading to phosphorylation of IκB by IKK, resulting in IκB ubiquination and degradation. NF-κB
proteins like p50 and p65 are then free to translocate to the nucleus, bind to their DNA response elements, and activate a wide variety of inflammatory response target genes (27–29). Consistent with these data, we previously demonstrated that inhibitors of NF-κB, proteins like p50 and p65, bind to their DNA response elements, and activate a wide variety of inflammatory response target genes (27–29).

AP-1 plays an important role in the transcriptional activation of inflammatory genes, including TNFα, IL-1β, and IL-6 (30–32). Because AP-1 activity is activated by a complex network of signaling pathways that involve JNK, p38, and ERK (33), this study examined whether α- and γ-MG inhibited LPS-induced phosphorylation of these MAPK. α- and γ-MG attenuated LPS-mediated phosphorylation of JNK, p38, and ERK. γ-MG was more effective than α-MG on an equimolar basis. Phosphorylation of c-Jun is a downstream target of JNK and a component of the transcription factor AP-1 (34). We demonstrated that γ-MG was more effective than α-MG in preventing the activation by LPS of c-Jun and AP-1 activity. These data suggest that suppression of AP-1 contributes to the decreased expression of inflammatory cytokines in α- and γ-MG-treated adipocytes.

Another possible mechanism of action by which MG prevents activation of inflammatory signaling is by blocking LPS-mediated increases in the levels of intracellular calcium [Ca^{2+}], or reactive oxygen species (ROS). Indeed, MG has been reported to prevent elevations in [Ca^{2+}], and prooxidants (35,36). Previous studies have shown that increased levels of [Ca^{2+}], or ROS cause activation of MAPK, NF-κB, and inflammatory cytokine expression (37,38). MG has 1 more hydroxyl group than α-MG. Therefore, it is possible that the extra hydroxyl group on γ-MG increases its ability to neutralize ROS, thereby reducing inflammatory signaling to a greater extent than α-MG. Thus, MG may attenuate LPS-mediated inflammation and insulin resistance by decreasing the levels of [Ca^{2+}], and ROS in human adipocytes. Future studies are needed to confirm these possibilities.

We demonstrate that γ-MG effectively prevents LPS-mediated insulin resistance in human adipocytes. Notably, γ-MG attenuated the suppression by LPS of the mRNA levels of PPARγ and adiponectin, a WAT-specific PPARγ target gene. Circulating levels of adiponectin are diminished in individuals who are obese or have type 2 diabetes (39) and are inversely correlated with risk factors for cardiovascular risk, including elevated C-reactive protein levels (40,41). Low adiponectin levels are also associated with dyslipidemia (42), hyperglycemia (43), hypertension (44), and coronary artery disease (45). Conversely, increasing adiponectin levels are associated with a reduction in body weight (46) and improved insulin sensitivity via AMP kinase activation (47–49). γ-MG may increase the levels of adiponectin, a PPARγ target gene, by reducing the activation of NFκB (50) or MAPK (51), which hinder PPARγ DNA-binding affinity or transcriptional activation. Taken together, our data suggest that γ-MG improves insulin sensitivity.

Collectively, these data demonstrate that γ-MG is more effective than α-MG in inhibiting the activation by LPS of TLR signaling, tenably by attenuating MAPK, NF-κB, and AP-1 activity that induce the expression of inflammatory genes, including TNFα, IL-1β, IL-6, IL-8, and MCP-1. Furthermore, γ-MG prevents the suppression by LPS of insulin-stimulated glucose uptake, possibly by preventing the suppression of PPARγ and adiponectin gene expression in primary cultures of human adipocytes. In vivo studies are needed to determine the ability of MG to recapitulate these in vitro findings.

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**Literature Cited**


α- and γ-Mangostin attenuate lipopolysaccharide-induced inflammation 1189
8. Tsukumo DML, Carvalho-Filho MA, Carvalheira JBC, Prada PO, Mandrup S, McIntosh MK. Conjugated linoleic acid (CLA) induces transport of a-mangostin from Garcinia mangostana (Mangosteen) using simulated digestion and Caco-2 human intestinal cells. Mol Nutr Food Res. In press 2009.


