Anti-Inflammatory Effects of Isoflavones are Dependent on Flow and Human Endothelial Cell PPARγ

Balu K. Chacko, Robert T. Chandler, Tracy L. D’Alessandro, Ameya Mundhekar, Nicholas K. H. Khoo, Nigel Botting, Stephen Barnes, and Rakesh P. Patel

Abstract

The mechanisms by which isoflavones protect against inflammatory vascular disease remain unclear. Our previous observations suggest that one mechanism involves inhibition of monocyte-endothelial cell interactions in a process that is absolutely dependent on flow. The molecular mechanisms involved and the effects of structurally distinct isoflavones on this process are not known and are investigated herein. Using static and flow-dependent monocyte adhesion assays, our data show that exposure of endothelial cells to biologically relevant concentrations of isoflavones inhibits subsequent TNF-α induced monocyte adhesion only during flow. This inhibition involved activating endothelial PPARγ by stimulating promoter sequences containing the PPARγ response element by isoflavones and attenuating antiadhesive effects by siRNA targeting of PPARγ. A comparison of structurally distinct isoflavones suggested a critical role for the A-ring. Using chlorinated derivatives of daidzein, a key structural requirement for PPARγ agonist activity appears to be the presence of the 7-OH group and the lack of chlorine at the 6- or 8-positions in the A-ring. Collectively, these data support 1) a novel flow-dependent anti-inflammatory mechanism for PPARγ ligands in vascular endothelial cells and 2) exemplify the current concepts of nutrients modulating disease via regulating specific cell signaling pathways. J. Nutr. 137: 351–356, 2007.

Introduction

Both experimental and epidemiological studies have highlighted the potential for dietary isoflavones to prevent atherosclerosis and other chronic diseases in which inflammation plays a key role (1–7). In human-based studies, consumption of isoflavones is associated with improved vascular function and decreased markers of oxidative damage (7–11), observations that support the general conclusion that isoflavones are a class of polyphenols with the capacity to protect the vasculature against inflammation-induced toxicity (12,13). Despite these concepts and the fact that isoflavones are widely consumed in botanical-based supplements, an understanding of the molecular mechanisms by which these compounds may exert effects in the vascular compartment remains poor.

Recent studies promote the concept that low, biologically relevant concentrations of isoflavones modulate cell signaling processes that impact on vascular disease. Genistein, a primary constituent of many isoflavone preparations, is as efficient as 17β-estradiol in binding to the β-isofrom of estrogen receptors and may explain the effects of this isoflavone on stimulating NO-dependent vasodilation (14). Furthermore, isoflavones are ligands for the nuclear receptor/transcription factors PPARα and γ (15). Genistein and daidzein stimulated PPARγ-dependent gene transcription in macrophages (4), with similar results observed for genistein in a preosteoblastic cell line (16). In vivo studies have shown that antidiabetic effects and lipid lowering effects of isoflavones also may be mediated by PPARγ and/or PPARα (4). In the context of atherosclerosis specifically, little is known about the vascular endothelial effects of PPARγ activation by isoflavones. Previous studies have documented that endothelial activation of PPARγ by synthetic ligands is anti-inflammatory by increased NO-bioavailability, inhibition of cytokine-dependent proinflammatory adhesion molecule expression, and subsequent leukocyte adhesion (17–19). Monocyte adhesion to the endothelium is an early and critical step in inflammation and the development of atherosclerotic lesions, and therefore represents a potentially important target of dietary isoflavones that, in turn, inhibit lesion development.

Leukocyte-endothelial interactions are characterized by the sequential and distinct events of tethering, rolling, firm adhesion, and then transmigration to the subendothelial region of the vessel. An important determinant of these interactions is the hydrodynamic forces associated with blood flow. In vivo, actively interacting leukocytes must overcome the shearing forces...
associated with blood flow to make close contact with the endothelium. In addition, chronic exposure of endothelial cells to shear stress is anti-inflammatory and may explain why atherosclerotic lesions are initiated at relatively low shear stress or turbulent flow sites in the vasculature. Our recent studies demonstrated that exposure of endothelial cells to genistein at low biologically relevant concentrations (≤1 μmol/L) inhibits leukocyte rolling and adhesion to TNF-α activated endothelial cells, representing a potent anti-inflammatory action of these compounds (20). Importantly, these effects were only observed in the presence of acute flow; no antiadhesive effect of genistein was observed if monocyte-endothelial cell interactions were determined under static conditions. Our data also indicated that neither estrogen receptors nor reactive species scavenging were important, and that the mechanism did not involve modulation of adhesion molecule expression but did suggest a role for PPARγ. Herein, we investigate the role of PPARγ in mediating anti-inflammatory effects of isoflavones during flow and evaluate potential structural motifs on isoflavones that may mediate these effects.

Materials and Methods

Human umbilical vein endothelial cells (HUVEC) and human acute monocytic leukemia cell line (THP-1) were purchased from ATCC or Clonetics. Daidzein, Biochanin A, and Equol were purchased from Indofine Chemicals. Genistein and chlorinated analogs of daidzein were prepared as previously described (21). Purity was assessed by MS/MS. The structure of all isoflavones used in this study is shown in Figure 1. Cell Tracker Green (CMFDA, C2925) or BCECF-AM fluorescent dyes were purchased from Molecular Probes. TNF-α and RPMI 1640 were purchased from Sigma Chemical. Endothelial growth medium (EGM) and the supplements (CC-3124) were purchased from Cambrex Biosciences. Rosiglitazone and GW 9662 were purchased from Cayman Chemicals. The nucleofection reagents and the green fluorescent protein (GFP) plasmid were purchased from Amaxa Biosystems. All other chemicals used in this study were of analytical grade.

Cell culture and viability. HUVEC were used between passages 3 and 7 and cultured as previously described (20). All experiments were performed within 1 d of cells reaching confluency. THP-1 cells were maintained in RPMI 1640 as previously described (20). For adhesion experiments under static or flow conditions, monocytes were labeled with cell tracker green (1 μmol/L) as described previously (20). Endothelial cells were treated with either TNF-α or different isoflavones (1 μmol/L, 16 h) as shown, washed with sterile warm PBS (2 times), and then used in adhesion assays. Due to varying specific activities of TNF-α from one batch to another, the concentrations that increased monocyte adhesion to endothelial cells by 50% under static conditions were determined (varied between 2 and 10 μg/mL) and those concentrations were used in this study.

Static adhesion assay. HUVEC were grown in 48-well plates and treated with isoflavones (1 μmol/L) for 16 h and during the last 4 h were coincubated with or without TNF-α. The percentage of monocyte adhesion under static conditions was then measured as previously described (20) using a final monocyte-endothelial cell ratio of 6:1 for 30 min.

In vitro flow assay. HUVEC were cultured in 35 mm dishes and treated with vehicle or isoflavones (1 μmol/L, 16 h) and during the last 4 h were coincubated with or without TNF-α. Leukocyte rolling and firm adhesion during flow was then determined as previously described (20) using the Glycotech flow chamber system and at flow rates of 300 μL/min (37°C) corresponding to a wall shear rate (or shear stress that the endothelial cells experience) of 1.5 dynes/cm². Cells were viewed on a Leica inverted fluorescence microscope equipped with a Hamamatsu Orca ER digital CCD camera (Compix) and real-time images of each field captured at 33 frames/s for 2 min, and the resulting time-lapse images were analyzed by motion tracking analysis using Automated Image Capture and Motion Tracking and Analysis software (Simple PCI, Compix). Any cell that did not move for 5 s or more was considered firmly bound and numbers calculated per min of data acquired.

PPRE reporter assay. Activation of PPARγ-dependent genes was assessed by evaluating the ability of isoflavones to stimulate the PPARγ-promoter responsive element (PPRE) linked to the luciferase gene. For these studies, HUVEC were transiently transfected with a CD36 reporter construct with (−273) or without (−261) its PPRE (transcription start site +1) (22) and inserted into pGL3b vector (Promega) (2 μg) (both kindly provided by Dr. Tom McIntyre) using Nucleofector according to manufacturer’s instructions. HUVEC were cotransfected with GFP or β-galactosidase (0.5–1 μg) to control for transfection efficiency. HUVEC were treated after transfection with isoflavones (1 μmol/L) or rosiglitazone (2 μmol/L) in EGM complete medium with 2% fetal bovine serum (FBS) for 16 h. All transfection experiments with peroxisomal proliferator response element (PPRE)-luciferase constructs included a luciferase control vector, pGL3b, as a negative control and the data were normalized to either β-galactosidase or GFP.

Measurement of luciferase activity. After treatment, cultures were rinsed twice with PBS, lysed with passive lysis buffer (Promega), centrifuged at 16,000 × g for 1 min, and the supernatants were collected. Luciferase activity was measured using Luciferase Assay Reagent (Promega) according to manufacturer’s directions. Transfection efficiency was determined by measuring β-galactosidase activity (Promega) at 420 nm using o-nitrophenyl β-D-galactopyranoside (Sigma) as the substrate. Alternatively, the GFP fluorescence (excitation 485 nm, emission 535 nm) was measured to control for transfection. Changes in reporter gene activity were calculated as a fold change in luciferase activity using the PPRE (−273) vs. PPRE-negative (−261) construct.

siRNA-dependent knockdown of PPARγ. HUVEC (passages 3–5) were grown (seed density of 12000 cells/cm²) in antibiotic-free EGM-2 medium containing 2% FBS until 50–60% confluence. Lipofectamine-siRNA transfection complexes were prepared by mixing 4 μL of Lipofectamine 2000 (Invitrogen) in 500 μL of OPTI-MEM with an appropriate amount of PPARγ siRNA [sequence: 5’ AUUGGAGAC-CCUCCCAUCUG 3’ (Qiagen)], previously shown to be effective in downregulating PPARγ in endothelial cells (23) to reach a final concentration of 300 nmol/L. The siRNA-lipofectamine complex was incubated at room temperature for 20 min and then added by drops into 1 well of a 6-well plate (2 mL volume) followed by gentle mixing. After 8 h the medium was changed to EGM containing 2% FBS. After 40 h

Figure 1 Structures of isoflavones used in this study.
HUVEC were treated with genistein and adhesion of monocytes determined as described above. In parallel incubations, cell lysates were collected after 40 h and percentage knockdown of PPARγ was determined by Western blot using 20 μg protein and separated by SDS-PAGE and wet-transfer to nitrocellulose membranes. Membranes were probed with anti-human PPARγ monoclonal antibody (SantaCruz Biotechnology) and developed using SuperSignal West Dura chemiluminescent substrate (Pierce Biotechnology). Band intensities were quantified using Scion Image software and normalized to β-actin. In parallel, controls using an equal amount of nonsilencing siRNA (sequence: 5’ CUUACGCUGAGUACUCGA 3’) and RNA-free lipofectamine control were also performed.

Statistical methods. In vitro flow and static adhesion experiments were conducted in triplicate and repeated at least 3 times. For in vitro flow experiments, data are plotted as fold changes relative to TNF-α. Significance was assessed using Student’s t test (for data in Fig. 2) and ANOVA (for data in Figs. 3–5) with post hoc analysis using Tukey test. Significance was set at a value of P < 0.05.

Results

Genistein mediated inhibition of monocyte adhesion during flow is dependent on PPARγ. To test whether genistein-mediated inhibition of monocyte adhesion during flow is PPARγ-dependent, an siRNA-based approach was used. siRNA effectively decreased PPARγ levels by ~75% compared with scrambled (nonsilencing) siRNA and empty vector controls (Fig. 2A). The addition of genistein to control or nonsilencing siRNA-treated cells significantly inhibited monocyte adhesion during flow (Fig. 2B). This inhibition is completely reversed, however, in cells treated with PPARγ siRNA.

Isoflavones activate PPARγ-dependent gene transcription in endothelial cells. To assess the PPARγ agonist activity of genistein and other structurally distinct isoflavones, HUVEC were transiently transfected with a plasmid containing the PPRE or corresponding control plasmids (empty or containing luciferase without PPRE). Isoflavones were then added to test their ability to stimulate PPARγ-dependent gene transcription. The synthetic PPARγ ligand rosiglitazone (positive control) and all the isoflavones tested, but not vehicle controls, increased PPRE-linked luciferase activity (i.e., no isoflavone treatment) cells transfected with PPRE containing plasmid (Fig. 3). Genistein increased luciferase activity to >3-fold that of the control. Relative to genistein, the effects of biochanin A were similar, whereas daidzein and equol activated PPARγ to a lesser extent. Importantly, genistein did not increase luciferase in cells transfected with PPRE-negative (−261) plasmids (control plasmid).

Isoflavones inhibit leukocyte-endothelial adhesion during flow. To test if PPARγ activation is translated into a functional inhibition of monocyte adhesion, the effects of these polyphenols on static- and flow-dependent monocyte adhesion was determined. Under static conditions, none of the isoflavones tested affected TNF-α induced THP-1 adhesion (Fig. 4A). However, in the presence of flow, all isoflavones inhibited monocyte adhesion consistent with the hypothesis that PPARγ plays a critical role in this inhibition (Fig. 4B).

Chlorination of daidzein modulates their ability to activate PPARγ and inhibit monocyte adhesion. We have shown that isoflavones can be chlorinated by hypochlorous acid formed during the phagocytic respiratory burst and that, in turn, this modification alters the antioxidant activity of these compounds (21,24). To test if chlorination affects PPARγ activation, and to

![Figure 2](image-url) Genistein inhibits monocyte adhesion during flow via endothelial PPARγ. siRNA decreased PPARγ in HUVEC (Panel A) and reversed the anti-adhesive effects of genistein (Panel B). A representative Western blot is shown (A). Quantitative data are expressed as fold of the control and are means ± SEM, n = 3–5. *Different from control, P ≤ 0.05. Data are expressed as fold of TNFα and are means ± SEM, n = 3–5 (B). *Different from TNFa, P ≤ 0.05.

![Figure 3](image-url) Isoflavones activate PPARγ promoter activity in HUVEC transfected with PPRE-reporter plasmids. Data are expressed as fold of the control (i.e., no isoflavone treated cells) and are means ± SEM, n = 3–6. Also shown are vehicle (methanol and DMSO for isoflavones and rosiglitazone, respectively) controls and effects of genistein in cells transfected with PPRE-negative plasmids. *Different from control, P ≤ 0.005.

![Figure 4](image-url) Isoflavones inhibit monocyte adhesion to TNF-α activated HUVEC only in the presence of flow (Panel B) but not under static conditions (Panel A). Data are expressed as fold of control (i.e., no treatment for Panel A and TNFα for Panel B) and are means ± SEM, n = 6–12. *Different from control, P ≤ 0.005, #Different from control, P ≤ 0.02.
gain further insights into the structural determinants that regulate isoflavone activation of PPARγ, specific chlorinated isomers of daidzein (see Fig. 1) were tested. These isomers have been synthesized to purity and, importantly, are formed upon exposure of the parent isoflavone to cellular-derived hypochlorous acid (21). Chlorination of daidzein at the 6- or 8-position ablated its ability to activate PPARγ-dependent transcription, whereas chlorination at the 3’ position had little effect (Fig. 5A). Moreover, these effects on PPARγ agonist activity were reflected functionally on monocyte adhesion. Specifically, modification at the 6- or 8-position also reversed the inhibitory effects of daidzein on monocyte adhesion during flow, whereas chlorination at the 3’-position had no effect, with 3’-Cl-daidzein inhibiting flow-dependent monocyte adhesion to TNF-α-activated HUVEC to a similar extent compared with native daidzein (Fig. 5B).

**Discussion**

Many studies have documented an association between the consumption of isoflavones and protection against cardiovascular disease. However, a more recent meta-analysis of controlled trials concluded that isoflavones (within the context of soy-based diets) confer only minimal cardiovascular benefits (25). These conclusions are based primarily on the effects of isoflavone consumption on cholesterol-lowering effects. However, few insights as to the potential effects of isoflavones on other elements of vascular disease were provided. This is critical because isoflavones exhibit antioxidant effects in vitro (26–28) and in vivo (7,8) and have more recently been shown to modulate vascular cell function that would limit vascular disease. Potential mechanisms for the latter include increasing nitric oxide bioavailability (9–11) and stimulating anti-inflammatory signaling pathways (12,13). The present data supports the latter concept, forwarding a critical role for isoflavones as inhibitors of inflammatory interactions between monocytes and endothelial cells. Monocyte rolling and adhesion to endothelial cells are among the early steps of the inflammatory cascade that ultimately leads to development of atherosclerotic lesions. We have shown that isoflavones at low biologically relevant concentrations (discussed below) do not modulate adhesion of monocytes to endothelial cells under static conditions [Fig. 4A and (20)]. However, monocyte adhesion in vivo occurs in the presence of flow and associated physical forces. Moreover, flow itself is a critical regulator of endothelial function with shear stress being shown to exhibit anti-inflammatory effects through multiple acute and chronic mechanisms, which underscores the importance of incorporating flow into experimental studies testing leukocyte-endothelial adhesion. Interestingly, when assessed during flow, isoflavones significantly inhibited TNF-α induced monocyte adhesion. The experimental protocol used suggests that isoflavones modulate how endothelial cells respond to inflammatory stimuli in the presence of physical forces associated with acute flow. It is important to distinguish this from chronic shear stress–mediated effects. In summary, our data suggest that long-term exposure to isoflavones regulate how endothelial cells respond to inflammatory stimuli, which are only functionally observed in the presence of flow (i.e., monocyte adhesion).

These antiadhesive effects of isoflavones are not mediated by their potential antioxidant activity, estrogenic potential, ability to inhibit tyrosine phosphorylation, nor by affecting expression of the adhesion molecules [i.e., endothelial selectin, vascular cell adhesion molecule-1 (VCAM-1), intercellular cell adhesion molecule-1 (ICAM-1), or platelet endothelial cell adhesion molecule-1 (PECAM-1)], typically involved in mediating monocyte-endothelial cell interactions (20). These data do not exclude a role for other adhesion molecules nor potential effects of genistein on post-translational regulation of adhesion molecule function. These possibilities are currently under investigation.

Our study focused on elucidating the molecular mechanisms involved and demonstrated that, using both reporter assays and siRNA targeting, PPARγ plays a critical role. These data are consistent with previous reports documenting a PPARγ agonist activity of isoflavones in adipocytes and macrophages (4,15,16). The PPARγ function in the endothelium and PPARγ agonist activity of isoflavones are not extensively characterized. It is important to note that synthetic/endoogenous ligands of PPARγ also prevent inflammatory monocyte-endothelial interactions during flow (29) similar to the effects reported herein. Moreover, all tested isoflavones that inhibited monocyte adhesion also activated PPARγ binding to its promoter response element and subsequent transcription. Furthermore, siRNA, targeted to PPARγ, but not scrambled siRNA, reversed genistein-mediated inhibition of monocyte adhesion. Recent reports suggest that 8 h of exposure to shear stress can activate PPARγ in endothelial cells (23) which could account for the effects of siRNA observed herein. However, in our studies, endothelial cells are exposed only to acute shear stress (for 1–2 min), precluding significant PPARγ-dependent transcription due to shear stress alone. Taken together, these data support 1) a critical role for endothelial PPARγ in modulating monocyte rolling and adhesion and 2) the potential importance of endothelial PPARγ as a molecular target for the anti-inflammatory effects of isoflavones.

Another critical variable in any effect of isoflavones is their specific composition within foods and commercially available botanical-based supplements, which varies widely and may contribute to disparate biological effects as discussed above. The issue is further complicated with the recognition that isoflavone metabolism involves glucuronidation, sulfation, and potential modification by reactive chlorinating and nitrating agents, and will depend on the specific composition of gut microflora and the degree of inflammatory stress (30,31). Our study demonstrates that structurally distinct isoflavones are able to inhibit monocyte adhesion to endothelial cells through the activation of PPARγ-dependent pathways. Furthermore, these effects are observed at low biologically relevant concentrations, which, for genistein can range between 0.3 and 0.8 μmol/L in the circulation (32–34).

The isoflavones tested in Figures 3 and 4 vary significantly in their antioxidant activities, but they all stimulated.
PPARγ-dependent gene transcription (Fig. 3) and inhibited monocyte adhesion (Fig. 4B). The structures (Fig. 1) of genistein, daidzein, biochanin A, and equol show that the hydroxyl group at the 7-position on the A-ring is a common structural feature of these isoflavones. The 5-hydroxy-ketone moiety is absent in equol and daidzein. In the case of biochanin A, methylation of the 4′-OH occurs. The fact that all the isoflavones displayed PPARγ agonist activity indicates a central role for the 7-OH group on the A-ring in this process. Data shown in Figure 3 would also suggest a role for the hydroxyl group on the 5-position of the A-ring because biochanin A and genistein (which both contain this group) showed greater PPARγ agonist activity compared with daidzein or equol (which lack this group).

To further determine the structural elements involved, we tested a variety of chlorinated daidzein derivatives. Chlorination at the 3′-position did not alter PPRE-dependent luciferase expression or effects on monocyte adhesion. However, modification at the 6- or 8-position on the A-ring attenuated these effects of daidzein. Together with the structure-activity insights discussed above, these data suggest that the isoflavone A-ring is critical in mediating specific interactions with PPARγ. We showed that chlorination of isoflavones occurs by phagocyte-derived hypochlorous acid, resulting in products with altered antioxidant properties toward lipid-based radicals (21). The present data extend these concepts to include modulation of endothelial PPARγ activity and suggest that halogenation on the A-ring will inhibit, but on the B-ring will have no affect on the anti-inflammatory properties.

In summary, this study demonstrates that isoflavones may protect against inflammatory vascular disease by inhibiting monocyte-endothelial cell adhesion through a mechanism that is absolutely dependent on flow and involves activation of endothelial PPARγ. However, phagocyte-derived hydroperoxic acid and chlorination of isoflavones at the 6- or 8-positions on the A-ring, but not the 3′-position in the B-ring, prevent the anti-adhesive effects of these isoflavones.

Acknowledgment
We thank Dr. Tom McIntyre (Lerner Research Institute, Cleveland, OH) for providing PPRE-con structs.

Literature Cited


