Oleanolic Acid Induces Prostacyclin Release in Human Vascular Smooth Muscle Cells through a Cyclooxygenase-2-Dependent Mechanism

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Abstract

Oleanolic acid is a triterpenoid that may contribute to the cardio-protective effects of olive oil. Our goal was to assess whether oleanolic acid could modulate eicosanoid biosynthesis and to determine the mechanism involved in this effect. Human coronary smooth muscle cells (SMC) were treated with oleanolic acid, eriodictyol, or hydroxytyrosol and eicosanoid release was measured by enzyme immunoassay. Cyclooxygenase (Cox)-1 and Cox-2 protein and messenger RNA levels were analyzed by Western blot and real-time PCR, respectively. Mitogen-activated protein kinase (MAPK) pathways were assessed using specific antibodies. Oleanolic acid induced prostaglandin I₂ (PGI₂) release by human coronary SMC, an effect that was prevented by celecoxib (a specific inhibitor of Cox-2). The increased PGI₂ was time- and dose-dependent and was associated to the up-regulation of Cox-2. No effects were observed on thromboxane A₂. Erythrodiol but not hydroxytyrosol upregulated Cox-2 expression and induced PGI₂ synthesis. Oleanolic acid induced an early phosphorylation of p38 MAPK and p42/44 MAPK but not c-Jun N-terminal kinase-1 (JNK-1). SB203580 (p38MAPK inhibitor) and U0126 ( MAPK kinase1/2 inhibitor) abrogated the upregulation of Cox-2 and PGI₂ release induced by oleanolic acid. A peptide inhibitor of JNK-1 (L-JNKI1) did not produce any effect. The induction of Cox-2 was preceded by an early activation of cAMP regulatory element-binding protein, a key transcription factor involved in Cox-2 transcriptional upregulation. Therefore, oleanolic acid contributes to vascular homeostasis by inducing PGI₂ release in a Cox-2-dependent manner. Oleanolic acid could be regarded as a bioactive molecule that may contribute to the beneficial effects of the Mediterranean diet. J. Nutr. 138: 443–448, 2008.

Introduction

The growing popularity of the Mediterranean diet is supported by a large body of epidemiological studies showing the protective effects of this diet against chronic diseases, including coronary heart disease [reviewed in (1)]. Many researchers are actively investigating olive oil, as an integral ingredient of the traditional Mediterranean diet, looking for bioactive components involved in the healthy advantages of this diet (2). In recent studies, natural triterpenoids contained in the "orujo" olive oil (oleanolic acid and erythrodiol) and methanolic extracts from other natural sources elicited vasorelaxation in isolated aortic rings from both normal and spontaneously hypertensive rats (3–5). The mechanism underlying these vasoprotective effects of oleanolic acid is not completely understood, although it seems to be at least in part endothelium mediated. In addition, synthetic triterpenoid analogs of oleanolic acid showed antiinflammatory properties preventing upregulation of both inducible nitric oxide synthase and cyclooxygenase (Cox)-2 in cultures of human macrophages and mouse RAW 264.7 cells stimulated with interferon-γ or LPS (6).

It recently became clear that Cox-2 plays a more complex role in the vascular system than the one earlier attributed to it. Certainly, Cox-2 has currently been associated with proinflammatory/proatherogenic stages due to its inducible nature and upregulation in monocyte-derived macrophages present in atherosclerotic lesions. However, Cox-2 may contribute to vascular prostaglandin I₂ (PGI₂) formation in healthy humans and data from both genetically modified mice and wild-type animal models indicate...
that Cox-2-derived PGI₂ prevents local thrombosis and neo-intima formation and contributes to the defensive mechanisms of the myocardium (7,8). We and others have shown that the vasoprotective properties of HDL could be related at least in part to their ability to induce PGI₂ release through Cox-2-dependent mechanisms in vascular smooth muscle cells (SMC) (9–12) and endothelial cells (13). The aim of this study was to determine whether the vasorelaxant effects of oleanolic acid were due to the potential regulation of Cox-2 and PGI₂ release in human coronary SMC.

Materials and Methods

Cell culture. Human coronary SMC were obtained and cultured as described (14). The study was approved by the Reviewer Institutional Committee on Human Research of the Hospital of Santa Creu i Sant Pau that conforms to the Declaration of Helsinki. Cells used in the experiments were between the 3rd and 5th passage. Vascular SMC cultures, arrested in a serum-free medium for 48 h, were treated with oleanolic acid, erthyrodiol, or hydroxytyrosol. Dimethyl sulfoxide, the vehicle for oleanolic acid and erthyrodiol, was routinely used as a control. As previously described (11,12), when inhibitors of signaling pathways were used, they were added 30 min before oleanolic treatment with U0126 (a MEK1/2 inhibitor) (Stressgen); SB203580 [a p38 mitogen-activated protein kinase (MAPK) inhibitor] (Oxford Biomedical Research); and L-JNKI1 [a c-Jun N-terminal kinase-1 (JNK-1) inhibitor; Tocris].

Lipoprotein isolation and characterization. Human plasma was collected from normal healthy volunteers. The study was approved by the Reviewer Institutional Committee on Human Research of the Hospital of Santa Creu i Sant Pau that conforms to the Declaration of Helsinki. HDL were isolated from a pool of fresh plasma samples as described (11,12). Lipoproteins were endotoxin free, as determined by the Limulus Amebocyte Lysate pyrogen testing system (Bioiwhttaker). HDL protein concentration was determined by the bicinchoninic acid protein assay (Pierce) and HDL cholesterol content by the cholesterol assay kit (Reflab). Vascular SMC were incubated with HDL cholesterol (0.78 mmol/L).

Eicosanoid determination. Culture media from vascular SMC cultures were collected and kept at –80°C. Levels of 6-keto-PGF₁α (the stable metabolite of PGI₂), prostaglandin E₂ (PGE₂), and thromboxane B₂ (TXB₂; the stable metabolite of TXA₂) were determined by an enzyme immunoassay (EIA) kit (Cayman Chemical) as described (11,12).

Western blot analysis. Vascular SMC cultures were washed twice with wash buffer (50 mmol/L HEPES, pH 7.4, 150 mmol/L NaCl, 100 mmol/L NaF, 10 mmol/L sodium pyrophosphate (NaPPi), 10 mmol/L EDTA, 2 mmol/L Na₃VO₄) and lysed with lysis buffer (wash buffer containing 1 mmol/L phenylmethylsulfonyl fluoride, 5 μmol/L leupeptin, 0.5% triton SDS) (15). Protein concentration was measured by the bicinchoninic acid protein assay (Pierce). Proteins were separated by SDS-PAGE, blotted onto nitrocellulose membranes, and dyed with Ponceau. Extra-cellular signal-regulated kinase1/2 (ERK1/2) was analyzed using 12.5% (30:0.3 acrylamide:bisacrylamide) gels that allow the detection of the 2 bands. Blots were incubated with antibodies against human Cox-2 (PG 27b, Oxford Biomedical), Cox-1 (160110, Cayman), human ERK1/2 (9102, Cell Signaling), human ERK1/2-P (phosphorylated form; 9106, Cell Signaling), human p38 MAPK (SC-7149, Santa Cruz Biotechnology), human p38 MAPK-P (phosphorylated form, M8177, Sigma), human JNK-1 (SC-474, Santa Cruz Biotechnology), human JNK-1-P (phosphorylated form, no. 9251, Cell Signaling Technology), human cAMP regulatory element-binding protein (CREB) phosphorylated in Ser133 (C9102; Sigma), or human CREB (C-21; Santa Cruz Biotechnology). Bound antibody was detected using the appropriate horseradish peroxidase-conjugated antibody (Dako). Signals were detected with the chemiluminescent detection system (Supersignal West Dura, Pierce) (11,12).

Real-time PCR. Total RNA from vascular SMC was isolated using RNeasy (Qiagen). Messenger RNA (mRNA) levels were determined by real-time RT-PCR. In brief, RNA was reverse transcribed with Taqman RT kit (Applied Byosystems) using random hexamers (16). Specific Taqman Assay-on-Demand real-time PCR primers and fluorescent probes (Applied Biosystems) were used for: Cox-2 (Hs00153133-m1) and Cox-1 (Hs00377721-m1). Glyceraldehyde-3-phosphate dehydrogenase (4326317E) was used as endogenous control to normalize results.

Other methods. To assess the possible cytotoxic effect of the different treatments, we analyzed cell morphology, cell viability, and cell apoptosis. Cell viability was analyzed by measuring the mitochondrial dehydrogenase activity with a commercial kit (XTT based assay for cell viability, Roche) (12). Treatments used in this study did not produce any cytotoxic effect.

Statistical analysis. Results are means ± SEM. A Stat View II (Abacus Concepts) statistical package for the Macintosh computer system was used for all analyses. Multiple groups were compared by 1-factor ANOVA (Unifrac). Post hoc multiple comparisons were made using the least significant difference test (LSD).

FIGURE 1 Effect of oleanolic acid on PGI₂ (A), PGE₂ (B), and TXA₂ (C) release in human coronary SMC. PGI₂ release (measured as its stable metabolite 6-keto-PGF₁α) from human coronary SMC stimulated (6 h) with oleanolic acid (50 μmol/L) or HDL cholesterol (0.78 mmol/L) in the presence of 10 μmol/L celecoxib (specific inhibitor of Cox-2) (A), PGE₂ levels measured by an EIA in cells treated as described in A (B), TXA₂ (measured as its stable metabolite TXB₂) and by EIA) levels in cells treated as described in A (C). Results are means ± SEM, n = 9. Means without a common letter differ, P < 0.05.
ANOVA followed by Fisher's protected least significant difference to assess specific group differences. Differences were considered significant at $P < 0.05$.

**Results**

Oleanolic acid induces prostacyclin release in human coronary SMC through a Cox-2-dependent mechanism. Human coronary SMC were incubated with oleanolic acid (50 μmol/L, 6 h) and PGI2 release was measured in the presence or absence of celecoxib (a Cox-2-specific inhibitor). Oleanolic acid significantly increased PGI2 release (>3.3-fold relative to controls) (Fig. 1A) and also modulated PGE2 release (90% greater than controls) (Fig. 1B), but PGE2 levels were 2 orders of magnitude lower than PGI2 levels. This effect that was prevented by celecoxib, suggesting the involvement of Cox-2 (Fig. 1C). Oleanolic acid did not modify the release of TXB2, the stable metabolite of TxA2 (Fig. 1C). Similar results were obtained with HDL cholesterol (0.78 mmol/L) used as a control. The increased PGI2 release by vascular SMC was associated to the upregulation of Cox-2 (both mRNA and protein levels) in a time- and dose-dependent manner (Figs. 2 and 3). Cox-1 protein (Figs. 2B and 3B) and mRNA levels (data not shown) were not modified. Erthrodiol, another triterpenoid present in the orujo olive oil, also induced both Cox-2 expression and PGI2 release in vascular SMC (Fig. 4). The effect of erthrodiol on Cox-2 expression was more transient than that produced by oleanolic acid and the magnitude of the effect on both Cox-2 mRNA levels and PGI2 release was lower than that produced by equivalent concentrations of oleanolic acid. In contrast, equivalent concentrations of hydroxytyrosol, a polyphenol present in the olive oil, neither induced Cox-2 nor modified PGI2 release (data not shown).

Oleanolic acid upregulates Cox-2 through the activation of MAPK pathways. Oleanolic acid induced the early activation (5 min after stimulus) of p38 MAPK and p42/44 MAPK (ERK1/2) but not JNK-1 (Fig. 5). Activation of p38 MAPK increased over time and was maximal at 1 h (Fig. 5A); by contrast, the activation of ERK1/2 was more transient (Fig. 5B), peaked at 15 min, and then decreased, although a significant activation was present even at 1 h after stimulus. These results suggest that both p38 MAPK and ERK1/2 could be involved in the upregulation of Cox-2 induced by oleanolic acid. To determine the contribution
of these MAPK pathways to Cox-2 upregulation and PGI2 synthesis induced by oleanolic acid, we analyzed the effect of cell-permeable-specific MAPK inhibitors. SB203580 (a p38 MAPK inhibitor), which efficiently prevented oleanolic acid-induced p38 activation (Fig. 5A), significantly reduced Cox-2 upregulation (both mRNA and protein levels) and PGI2 release (Fig. 6). Similarly, U0126 (an ERK1/2 inhibitor), which efficiently prevented oleanolic acid-induced ERK1/2 activation (Fig. 5B), significantly reduced Cox-2 upregulation (both mRNA and protein levels) and PGI2 release (Fig. 6).

Finally, we analyzed the potential involvement of CREB, a key transcription factor in Cox-2 transcriptional regulation, in the induction of Cox-2 by oleanolic acid. Oleanolic acid induced CREB activation early (phosphorylation in Ser 133), 5 min after stimulus, reaching a maximal induction at 15 min (Fig. 7A). To further determine the involvement of CREB in the upregulation of Cox-2, we analyzed whether MAPK inhibitors prevent CREB activation. Indeed, specific inhibitors of both p38 MAPK (SB203580) and ERK1/2 (U0126) prevented the activation of CREB (Fig. 7A). Figure 7B summarized the pathways involved in Cox-2 expression/PGI2 release induced by oleanolic acid.

Discussion
Numerous epidemiological studies have documented an inverse relationship between Mediterranean diet intake and coronary heart disease; however, the mechanisms by which specific dietary components contribute to this protective effect are not entirely understood. In previous studies, we have shown that oleanolic acid, a natural triterpenoid contained in the orujo olive oil, elicits vasorelaxation in isolated aortic rings from both normal and spontaneously hypertensive rats through a mechanism depending at least in part on endothelium-derived vasorelaxant molecules (3–5). We now report that in human coronary SMC, oleanolic acid is able to induce PGI2 release through a mechanism involving Cox-2 upregulation via MAPK signaling pathways. This is the first study to our knowledge showing the ability of oleanolic acid to modulate PGI2 synthesis by vascular SMC.

For years, oleic acid, the major fatty acid in olive oil, has been considered to be mainly responsible for the healthy properties, in particular cardio-protective, of olive oil. However, oleic acid is also abundant in pork and chicken meats and certain studies indicate that its consumption is only slightly higher in Mediterranean countries than in the US (17). Therefore, oleic acid does not seem to be the sole component of olive oil that confers healthy properties to this food (18). Recently, minor components...
of olive oil have emerged as potential bioactive molecules involved in their healthy properties (19,20). These components could modify the size and composition of triglyceride-rich lipoproteins in humans (21), inhibit acyl-coenzyme A:cholesterol acyltransferase (22), modulate hepatic gene expression and exert atheroprotective properties in apolipoprotein E-deficient mice (23,24), alter cytokine secretion from human peripheral blood mononuclear cells (25), reduce blood pressure in animal models (26,27), and improve endothelial cell function (6,28), among other effects [reviewed in (20)].

Our results show that oleanolic acid is a strong inducer of PGI2 synthesis in human coronary SMC. This effect is completely prevented by celecoxib, indicating the dependence on Cox-2 activity. Oleanolic acid moderately increases the release of PGE2 and, interestingly, does not affect the release of TxA2, a vasoconstrictor eicosanoid. This pattern of modulation of eicosanoid synthesis by oleanolic acid is similar to that produced by HDL. In fact, the levels of PGI2 released by SMC treated with oleanolic acid (or HDL) were 100-fold higher than those produced by oleanolic acid at equivalent concentrations. On the contrary, hydroxytyrosol, a bioactive polyphenol present in olive oil, neither induces Cox-2 nor modifies PGI2 release. Therefore, our results suggest that upregulation of the Cox-2 pathway could be a property of triterpenoids not shared by other bioactive components of olive oil.

The upregulation of Cox-2 and the subsequent increase of PGI2 release induced by oleanolic acid are dependent on the activation of p38 MAPK and ERK1/2. In fact, specific inhibitors of these MAPK efficiently prevented PGI2 release and Cox-2 upregulation (both mRNA and protein levels). These pathways are similar to those activated in vascular SMC by other Cox-2 inducers such as HDL (11,12). Oleanolic acid did not induce JNK-1 and produced a more sustained activation of p38 MAPK (remained active 1 h after stimulus) than that produced by HDL (12). Indeed, HDL are complex particles that carry multiple bioactive components able to activate different cell signaling pathways and to modulate vascular function. The activation of signaling by oleanolic acid leads to the downstream activation of CREB, the key factor involved in Cox-2 transcriptional regulation by physiologically relevant agonists such as HDL and drugs such as statins (29,30).
Currently, there are no studies to our knowledge reporting plasma levels of oleic acid after the intake of olive oil. Dosages used in this study are similar to those previously reported by other authors in cell cultures or organ bath systems (3,4,31,32). At these dosages, which should be considered pharmacological, oleic acid is able to improve the balance of vasodilator/antiaggregant vs. vasoconstrictor/prothrombotic eicosanoids in human coronary SMC in culture. Oleic acid could be regarded as a bioactive molecule that could contribute to the beneficial effects of the Mediterranean diet. However, further studies are needed to confirm the relevance of these effects in humans habitually consuming olive oil, especially olive oils such as orujo with a high content of oleic acid.

In summary, oleic acid induces PG12 release by human coronary SMC in a Cox-2-dependent manner, activating mechanisms common to those involved in HDL-induced upregulation of Cox-2 that include MAPK signaling and CREB activation.

**Literature Cited**


