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# Eicosapentaenoic acid increases cytochrome P-450 2J2 gene expression and epoxyeicosatrienoic acid production via peroxisome proliferator-activated receptor $\gamma$ in endothelial cells

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**Summary**  $\omega$ -3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have beneficial effects on cardiovascular diseases. Cytochrome P-450 (CYP) 2J2 that is expressed in endothelial cells metabolizes arachidonic acids to biologically active epoxyeicosatrienoic acids (EETs) that possess anti-inflammatory and anti-thrombotic effects.

We studied the effects of EPA and DHA on the expression of CYP 2J2 mRNA by reverse transcription-polymerase chain reaction in cultured human umbilical vein endothelial cells and found that EPA, but not DHA, increased the expression of CYP 2J2 mRNA in a dose-dependent and a time-dependent manner. EPA-induced CYP 2J2 expression was significantly inhibited by pretreatment with a peroxisome proliferator-activated receptor (PPAR)  $\gamma$  antagonist, GW9662. EPA, but not DHA, caused a significant increase in cellular levels of 11,12-dihydroxyeicosatrienoic acid that is a stable metabolite of 11,12-EET, which was blocked by pretreatment with GW9662.

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These data demonstrate that EPA increases CYP 2J2 mRNA expression and 11,12-EET production via PPAR $\gamma$  in endothelial cells and indicate a novel protective role of EPA and PPAR $\gamma$  against vascular inflammation.

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## Introduction

Accumulating evidence has revealed that  $\omega$ -3 fatty acids, such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have beneficial effects, including lipid lowering, blood pressure lowering and anti-arrhythmic effects, on cardiovascular diseases [1,2]. EPA and DHA exert their biological effects by activating cell signaling pathways. It has been demonstrated that EPA increases the activity of peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ) [3]. PPAR $\gamma$ , a member of the steroid/retinoid nuclear receptor family of ligand-activated transcription factors, is expressed in vascular cells and inflammatory cells [4]. The activation of PPAR $\gamma$  appears to have anti-atherogenic effects through the inhibition of endothelial adhesion molecule expression, proliferation of vascular smooth muscle cells, and modified low-density lipoprotein receptor expression in macrophages [5–7].

Cytochrome P-450 (CYP) 2J2 that is expressed in vascular endothelial and smooth muscle cells and cardiomyocytes metabolizes arachidonic acid to biologically active epoxyeicosatrienoic acids (EETs) [8]. 11,12-EET produced by CYP 2J2 has anti-inflammatory properties such as prevention of leukocyte adhesion to endothelial cells by inhibiting nuclear factor- $\kappa$ B that stimulates the expression of proinflammatory gene products in endothelial cells [8]. 5,6-, 8,9-, 11,12-, and 14,15-EETs have vasodilatory effects by polarization and relaxation of vascular smooth muscle cells. EETs also are reported to have anti-thrombotic effects by upregulating tissue plasminogen activator and anti-migratory effects against vascular smooth muscle cells through decreasing the production of reactive oxygen species [9]. Spiecker et al. demonstrated that a polymorphism of the CYP 2J2 gene that results in decreased CYP 2J2 promoter activity and lower plasma concentrations of EET metabolites is independently associated with an increased risk of coronary artery disease [10]. Thus, it is suggested that CYP 2J2-induced EETs have protective effects against atherosclerosis and vascular remodeling.

In this study, we investigated the effects of EPA and DHA on the expression of CYP 2J2 mRNA by reverse transcription-polymerase chain reaction

(RT-PCR) in cultured human umbilical vein endothelial cells (HUVECs). EETs that are produced from arachidonic acids by 2C and 2J classes of CYP epoxygenases are quickly metabolized into stable metabolites, dihydroxyeicosatrienoic acids (DHETs) [8,10]. Therefore, cellular DHET levels are considered to be a surrogate marker of cellular EET levels. To investigate the effects of EPA and DHA on the epoxygenase activity to produce EET in endothelial cells, we studied the effects of EPA and DHA on cellular levels of 11,12-DHET in HUVECs by enzyme-linked immunoassay (ELISA). Also, the involvement of PPAR $\gamma$  in the signaling pathway that induces CYP 2J2 gene expression and 11,12-EET production in HUVECs was studied using a PPAR $\gamma$  antagonist, GW9662.

## Methods

### Materials

EPA and DHA were purchased from Sigma (St Louis, MO, USA). GW9662 was obtained from Cayman Chemical Co. (Ann Arbor, MI, USA).

### Cell culture

HUVECs purchased from Cambrex (Walkersville, MD, USA) were cultured in RPMI medium (Sigma) containing 20% fetal bovine serum, 25 units/ml heparin, 20 mg/ml endothelial cell growth supplement, 100 U/ml penicillin, and 100  $\mu$ g/ml streptomycin.

### Reverse transcription-polymerase chain reaction

Total RNA was isolated from cells with Isoagen (Nippon Gene, Toyama, Japan) following the manufacturer's protocol. cDNA synthesis was performed using 2  $\mu$ g total RNA and a RETROscript (Ambion, Austin, TX, USA) following the manufacturer's protocol. The specific primer sets for CYP 2J2 were 5'-GGACTCTCCTACTGGGCACT-3' and 5'-CTCCGAAGGTGATGGAGCAA-3' (Genbank accession no. U37143).  $\beta$ -Actin was amplified using Quantum RNA  $\beta$ -actin Internal Standards from Ambion.

After an initial denaturation step at 94°C for 5 min, the reactions underwent 27 cycles of 1 min at 94°C, 2 min at 65°C, and 3 min at 72°C. PCR products were electrophoresed on 2% agarose gels followed by ethidium bromide staining. The mRNA expression levels were normalized by  $\beta$ -actin expression and presented as the relative expression level compared to that of control cells.

### DHET measurement

HUVECs that were treated with EPA or control vehicle for 24 h in the presence or absence of GW9662 were scraped into tubes. Cells were sonicated in phosphate buffered saline containing triphenylphosphine. After pH adjustment with acetic acid, homogenized cells were extracted with ethyl acetate. After centrifugation, organic phase was evaporated, reconstituted with *N,N*-dimethyl formamide, and was subjected to ELISA assay. Commercially available ELISA kit to measure 11,12-DHET (Detroit R&D, Inc., MI, USA) was used following the manufacturer's protocol.

### Statistical analysis

Data from five independent experiments are shown as means  $\pm$  S.E. Statistical significance, calculated

using Mann–Whitney's *U* test and Wilcoxon signed-rank test, was taken as  $p < 0.05$ .

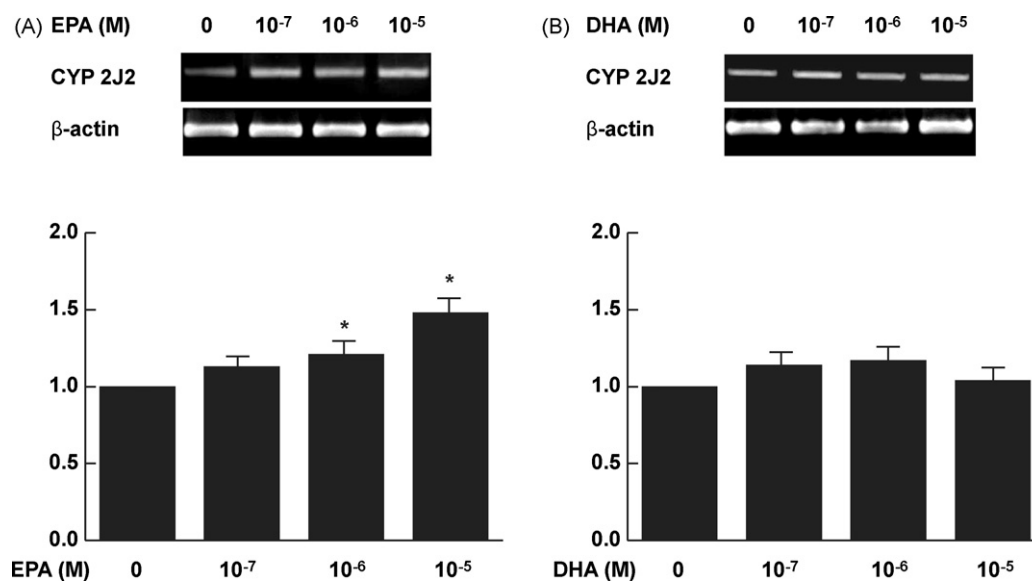
## Results

### EPA stimulates CYP 2J2 mRNA expression in endothelial cells in a dose-dependent manner

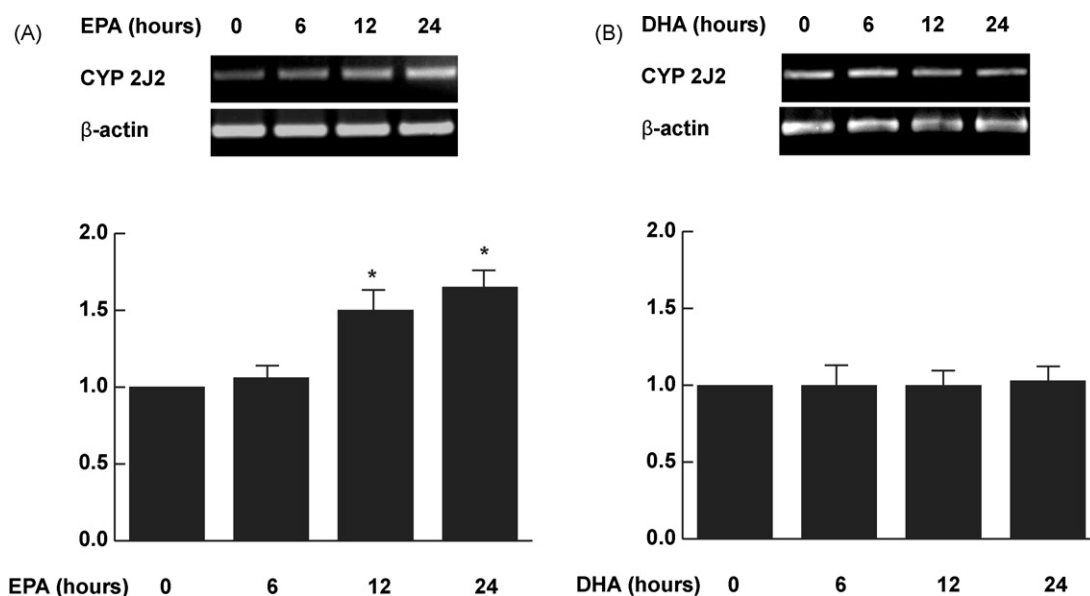
We investigated the effects of EPA and DHA on the expression of CYP 2J2 mRNA in endothelial cells by RT-PCR. HUVECs were treated with control vehicle, EPA, and DHA at indicated dose for 24 h. As shown in Fig. 1A,  $10^{-6}$  and  $10^{-5}$  M of EPA significantly increased CYP 2J2 mRNA expression in a dose-dependent manner. In contrast, DHA had no significant effects on CYP 2J2 mRNA expression in the same dose range (Fig. 1B). These data indicate that EPA, but not DHA, stimulates CYP 2J2 mRNA expression in endothelial cells in a dose-dependent manner.

### EPA stimulates CYP 2J2 mRNA expression in endothelial cells in a time-dependent manner

To study the time course of CYP 2J2 mRNA expression induced by EPA in endothelial cells, HUVECs



**Figure 1** Eicosapentaenoic acid (EPA) induces cytochrome P-450 (CYP) 2J2 mRNA expression in a dose-dependent manner. Total RNA extracted from human umbilical vein endothelial cells that were treated with EPA (A) and docosahexaenoic acid (DHA) (B) at indicated dose for 24 h was subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis for CYP 2J2 mRNA expression. The PCR products were analyzed by agarose gel electrophoresis (upper panels). The band intensity of CYP 2J2 was adjusted with the band intensity of  $\beta$ -actin, and data shown are the mean  $\pm$  S.E. of five independent experiments expressed as the fold increase compared with the value for control cells (lower panels). \* $p < 0.05$  vs. untreated control.



**Figure 2** Eicosapentaenoic acid (EPA) induces cytochrome P-450 (CYP) 2J2 mRNA expression in a time-dependent manner. Total RNA extracted from human umbilical vein endothelial cells that were treated with  $10^{-5}$  M of EPA (A) and docosahexaenoic acid (DHA) (B) for the indicated time was subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis for CYP 2J2 mRNA expression. The PCR products were analyzed by agarose gel electrophoresis (upper panels). The band intensity of CYP 2J2 was adjusted with the band intensity of  $\beta$ -actin, and data shown are the mean  $\pm$  S.E. of five independent experiments expressed as the fold increase compared with the value for control cells (lower panels). \* $p < 0.05$  vs. untreated control.

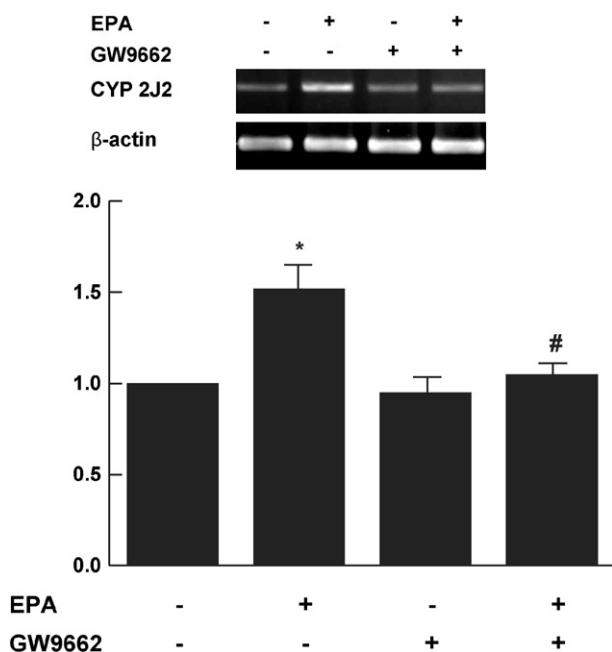
were treated with  $10^{-5}$  M of EPA for the indicated time. As shown in Fig. 2A, treatment with EPA for 12 and 24 h significantly increased CYP 2J2 mRNA expression compared to basal expression of CYP 2J2 mRNA in HUVECs. In contrast, DHA treatment showed no significant effects on CYP 2J2 mRNA expression in HUVECs (Fig. 2B). These data indicate that EPA, but not DHA, stimulates CYP 2J2 mRNA expression in endothelial cells in a time-dependent manner.

### Involvement of PPAR $\gamma$ in CYP 2J2 mRNA expression in endothelial cells

It has been shown that EPA induces PPAR $\gamma$  activation that results in gene expression [3]. To investigate the involvement of PPAR $\gamma$  in EPA-induced CYP 2J2 expression in endothelial cells, HUVECs that were pretreated with a PPAR $\gamma$  antagonist GW9662 or control vehicle were stimulated with EPA ( $10^{-5}$  M) for 24 h. As shown in Fig. 3, pretreatment with GW9662 significantly prevented the EPA-induced upregulation of CYP 2J2 mRNA expression in HUVECs. Pretreatment with GW9662 had no obvious effects on basal CYP 2J2 expression. These results indicate that PPAR $\gamma$  is involved in EPA-induced CYP 2J2 expression in endothelial cells.

### EPA stimulates EET production via PPAR $\gamma$ in endothelial cells

The epoxygenase activity of CYP 2J2 and 2C9 is demonstrated to metabolize arachidonic acid into EETs including 11,12-EET and 14,15-EET that have vasodilatory and anti-inflammatory properties [8]. Among EETs, 11,12-EET is the most potent biologically active metabolite. EETs are quickly metabolized into a stable metabolite DHETs including 11,12-DHET and 14,15-DHET. Therefore, cellular DHET levels are considered to be a surrogate marker of cellular EET levels [8,10]. To study the effects of EPA and DHA on the CYP epoxygenase activity to produce 11,12-EET in endothelial cells, we measured cellular 11,12-DHET levels by ELISA. Also, the involvement of PPAR $\gamma$  was examined using a pharmacological PPAR $\gamma$  antagonist GW9662. Cell lysates prepared from HUVECs stimulated with control vehicle, EPA, or DHA at indicated dose after pretreatment with a PPAR $\gamma$  antagonist GW9662 or control vehicle were subjected to ELISA assay to measure cellular 11,12-DHET levels. As shown in Fig. 4,  $10^{-5}$  M of EPA significantly increased cellular 11,12-DHET levels up to  $1.9 \pm 0.3$ -fold of control in HUVECs. DHA had no significant effects on cellular 11,12-DHET levels. An increase of cellular 11,12-DHET induced by EPA was abolished by pre-

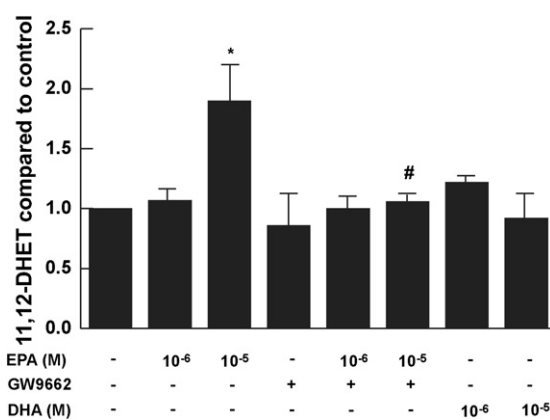


**Figure 3** Involvement of peroxisome proliferator-activated receptor (PPAR)  $\gamma$  in eicosapentaenoic acid (EPA)-induced cytochrome P-450 (CYP) 2J2 mRNA expression in endothelial cells. Total RNA extracted from human umbilical vein endothelial cells that were pretreated with a PPAR $\gamma$  antagonist GW9662 ( $10^{-7}$  M) and then stimulated with EPA ( $10^{-5}$  M) for 24 h was subjected to reverse transcription-polymerase chain reaction (RT-PCR) analysis for CYP 2J2 mRNA expression. The PCR products were analyzed by agarose gel electrophoresis (upper panel). The band intensity of CYP 2J2 was adjusted with the band intensity of  $\beta$ -actin, and data shown are the mean  $\pm$  S.E. of five independent experiments expressed as the fold increase compared with the value for control cells (lower panel). \* and # indicate  $p < 0.05$  vs. untreated control and EPA-treated cells, respectively.

treatment with GW9662. These data indicate that EPA, but not DHA, increases 11,12-EET production via PPAR $\gamma$  in endothelial cells.

## Discussion

The epoxygenases that synthesize EETs in endothelial cells are primarily CYP 2C9 and CYP 2J2 [8,11,12]. As EETs produced by CYP 2J2 have anti-inflammatory effects that are potentially vasoprotective, we studied changes in the expression of CYP 2J2 in cultured human endothelial cells and found that EPA, but not DHA, increases CYP 2J2 gene expression. In accordance with the increase in CYP 2J2 gene expression, cellular levels of 11,12-DHET that is a stable metabolite of 11,12-EET were increased in response to EPA in endothelial cells.



**Figure 4** Eicosapentaenoic acid (EPA) stimulates epoxyeicosatrienoic acid (EET) production via peroxisome proliferator-activated receptor (PPAR)  $\gamma$  in endothelial cells. Cell lysates prepared from human umbilical vein endothelial cells stimulated with EPA and docosahexaenoic acid (DHA) at the indicated dose after pretreatment with a PPAR $\gamma$  antagonist GW9662 or control vehicle were subjected to enzyme-linked immunoassay to measure cellular 11,12-dihydroxyeicosatrienoic acid (DHET) levels, as described in Methods. Data shown are the mean  $\pm$  S.E. expressed as the fold increase in cellular levels of 11,12-DHET in five independent experiments compared with the value for control cells. \* and # indicate  $p < 0.05$  vs. untreated control and  $10^{-5}$  M EPA-treated cells, respectively.

CYP 2C that produces vasodilatory EETs is implicated in the regulation of endothelial proliferation and angiogenesis [13,14]. So far, molecular mechanisms that are responsible for induction of CYP 2J2 and CYP 2C are not fully understood. CYP 2J2 and CYP 2C proteins rapidly and markedly decrease in cell isolation for cell culture. To differentially inactivate the isoforms of CYP 2J and CYP 2C have not been achieved. Future studies are necessary to determine the major source of EPA-induced EETs in endothelial cells. Our present study also provides novel findings that PPAR $\gamma$  mediates EPA-induced CYP 2J2 expression. The results in the present study indicate that EPA and DHA have differential effects on CYP 2J2 expression in endothelial cells. Omura et al. demonstrated that EPA, but not DHA, activates endothelial nitric oxide synthase and increases nitric oxide production in endothelial cells [15]. These data suggest that EPA and DHA stimulate differential signaling pathways probably depending on cell types.

It is likely that the effects of EETs are mediated by binding to postulated membrane receptors as well as by interacting directly with intracellular signaling molecules, transcription factors, and ion channels [16]. 11,12-EET is shown to activate a signaling pathway mediated by G $\alpha_s$  that is a compo-

ment of a heterodimeric GTP-binding protein [9]. On the other hand, EETs bind to intracellular proteins that contain fatty acid binding sites including PPAR $\gamma$  [17]. Interestingly, Huang et al. demonstrated that shear stress induces EPA-mediated EET production in endothelial cells [18]. Therefore, there is a possibility that 11,12-EET induced by EPA has additional effects on EPA-stimulated PPAR $\gamma$  activity.

PPAR $\gamma$  is reportedly expressed in endothelial cells, vascular smooth muscle cells, and human atherosclerotic lesions [4,19]. Previous studies have revealed anti-inflammatory effects of PPAR $\gamma$  agonists on endothelial cells such as inhibition of the expression of adhesion molecules, increased nitric oxide bioavailability, and repression of NADPH oxidase activity [20,21]. Xu et al. reported the binding of EPA to the ligand-binding domain of PPAR $\delta$  [22]. The structural homology among PPAR isoforms is divergent in the ligand-binding domain and the interaction between EPA and PPAR $\gamma$  is not yet determined [23]. It is reported that PPAR $\gamma$  mRNA expression was not changed by EPA treatment in certain types of cells [24]. We also confirmed that EPA did not significantly change PPAR $\gamma$  mRNA expression in HUVECs, suggesting that EPA increases PPAR $\gamma$  activity without upregulation of its mRNA (data not shown). Although further studies are necessary to investigate a possibility that EPA binds to PPAR $\gamma$  and mechanisms how EPA activates CYP 2J2 transcription, our present study provides novel evidence that EPA stimulates CYP 2J2 transcription via PPAR $\gamma$ .

In summary, our data indicate that EPA-induced CYP 2J2 expression and EET production in endothelial cells contribute to vasoprotective effects of EPA. Also, endothelial PPAR $\gamma$  activity appears to be required for EPA to stimulate CYP 2J2 expression, suggesting a possible therapeutic effect of a combination of EPA and synthetic PPAR $\gamma$  agonists for cardiovascular diseases.

## Conclusion

The present study demonstrates that EPA, but not DHA, increases CYP 2J2 mRNA expression and 11,12-EET production in endothelial cells via PPAR $\gamma$ , indicating a novel vascular protective effect of EPA.

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