Regression of pre-established atherosclerosis in the apoE$^{-/-}$ mouse by conjugated linoleic acid

S. Toomey*, H. Roche†, D. Fitzgerald* and O. Belton*[1]

*Department of Clinical Pharmacology, Royal College of Surgeons in Ireland, 123 St. Stephens Green, Dublin 2, Ireland, and †Department of Clinical Medicine, Trinity College, University of Dublin, Dublin 2, Ireland

Abstract

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid that has been shown to suppress the development of atherosclerosis in a rabbit model. We investigated whether CLA acts as a cyclo-oxygenase (COX) inhibitor or as an agonist of the peroxisome-proliferator-activator receptor (PPAR) γ in the ApoE$^{-/-}$ mouse model. *In vitro*, a 9-*cis*, 11-*trans* isomer of CLA inhibited prostaglandin formation and oxygen consumption by both isoforms of COX, with no evidence by MS of alternative products being generated. *In vivo*, supplementation with CLA was found to induce resolution of atherosclerosis. The effect of CLA *in vivo* could not be explained by COX inhibition alone, as urinary prostaglandin levels were unchanged in animals receiving CLA supplementation, and administration of selective COX inhibitors did not induce lesion regression. There was however induction of PPARγ, a known response to agonists of this nuclear orphan receptor.

Atherosclerosis

Atherosclerosis is a progressive disease of medium and large arteries characterized by the accumulation of lipids in inflammatory cells leading to foam-cell formation [1]. The disease is also characterized by cellular proliferation, platelet adherence and aggregation, and calcium deposition [2]. It is the primary cause of heart disease and stroke in the Western world, being responsible for 50% of all deaths. Many agents are used in the treatment of the disease; however, these are ineffective at reversing the process.

Eicosanoid biosynthesis

The prostaglandins (PGs), thromboxanes (TXs), leukotrienes and hydroxyeicosatetraenoic acids (HETEs) are termed eicosanoids, as they are derived form 20-carbon essential fatty acids that contain three, four or five double bonds. PGs are generated from arachidonic acid by the enzyme cyclo-oxygenase (COX) [3] (Scheme 1). Arachidonic acid is converted to the unstable intermediate PGG_2 by a COX reaction, where a proton is extracted and molecular oxygen is inserted. PGG_2 is converted into PGH_2 by a two-electron reduction catalysed by a spatially distinct peroxidase. In a final step, PGH_2 is reduced or rearranged by often tissue-specific isomerases and synthases to yield the prostanoids, including PGE_2, PGD_2, PGI_2 (prostacyclin) and TXA_2. These biologically active PGs act in a paracrine fashion through specific seven-transmembrane and G-protein-coupled receptors [3]. However, PGs may also act as intracellular signals since they are agonists of peroxisome-proliferator-activated receptors (PPARs), nuclear membrane proteins that heterodimerize with other proteins to form transcription factors.

Overview of COX

Two isoforms of COX have been identified: COX-1 and COX-2 [4]. COX-1 and COX-2 are the products of two different genes, situated on chromosomes 9 and 11 respectively [5]. The COX-1 gene encodes a 576-amino-acid protein, whereas the COX-2 gene encodes a protein product of 587 amino acids [6], with 60% identity between the two enzymes. Differences between the two isoforms are seen at the N-terminal signal-peptide region and a C-terminal 18-amino-acid insertion in the COX-2 polypeptide. The remaining sequence is very similar, with all residues essential for catalytic activity conserved [7]. The COX proteins consist of three distinct domains: an N-terminal epidermal-growth-factor domain, a membrane-binding motif and a C-terminal catalytic domain that contains the COX and peroxidase active sites. The two genes have distinct promoters. COX-1 lacks a TATA box, and instead has multiple transcription start sites [3], a feature commonly associated with 'housekeeping' genes. The promoter region of the COX-2 gene does contain a TATA box, 31 bases upstream from the transcriptional start site [8]. The COX-2 promoter contains a number of putative response elements including those for nuclear factor κB (NFκB)-binding protein, and a cAMP response element (CRE), that are common in highly regulated genes. This gives COX-2 the characteristics of an 'immediate-early'

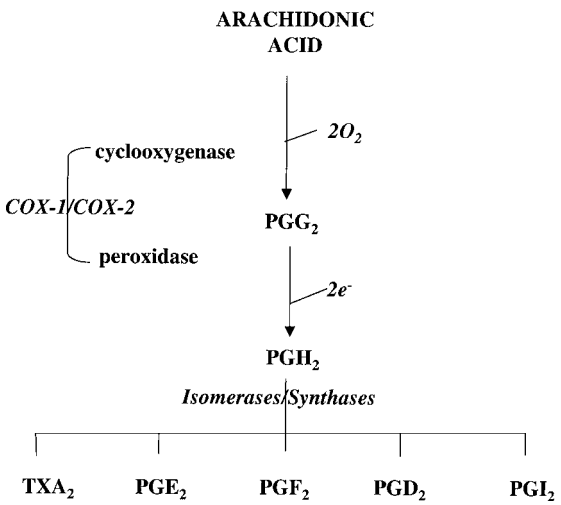
Key words: atherosclerosis, conjugated linoleic acid, cyclo-oxygenase (COX), eicosanoid, prostaglandin.

Abbreviations used: COX, cyclo-oxygenase; LDL, low-density lipoprotein; PG, prostaglandin; PPAR, peroxisome-proliferator-activated receptor; TX, thromboxane; VSMC, vascular smooth muscle cell.

[1]To whom correspondence should be addressed (e-mail obelton@rcsi.ie).

Scheme 1 | Generation of eicosanoids from arachidonic acid

Arachidonic acid is converted into PGG_2 by the COX activity of either COX-1 or COX-2, and into PGH_2 by the peroxidase activity of the enzyme. PGH_2 is converted by various tissue-specific isomerases and synthases into the biologically active PGs.



gene that is rapidly up-regulated during inflammation, for example.

Traditionally, COX-1 has been classified as the 'housekeeping' enzyme and COX-2 has been classified as the inducible isoform. However, this distinction is not entirely accurate. Although COX-1 is constitutively expressed by most cells and in virtually all tissues under normal physiological conditions [9], the enzyme is inducible in response to various stimuli and particularly at sites of inflammation [10]. COX-2 expression is strongly induced at sites of inflammation, in response to cytokines [11], growth factors [12], hypoxia [13], and free radicals [14]. However, COX-2 has been found to be expressed in some tissues, including brain [15] and kidney [16] under physiological conditions. COX-2 expression in the foetus has an important role in development. For example, mice that lack a functional COX-2 gene display renal dysplasia [17] and patent ductus arteriosus [18].

COX expression in the vasculature

Both endothelial cells and vascular smooth muscle cells (VSMCs) express COX, although endothelial cells contain >20 times more COX than VSMCs. In large vessels, PGI_2 is formed predominantly in the endothelial layer by COX-1 and COX-2, whereas the underlying VSMCs generate considerably less PG [19]. In small vessels, the principal product is PGE_2. In addition to normal tissue, COX-1 is expressed in increased amounts in atherosclerotic plaques, predominantly in VSMCs underlying the plaque, as well as in adjacent sections with normal morphology [20].

COX-2 is the predominant source of PGI_2 in man; however, the source of COX-2 is unknown [19]. *Ex vivo* studies show no evidence that COX-2 is expressed in normal blood vessels [21]. COX-2 is expressed in atherosclerotic plaques *ex vivo*, and the increase in expression is evident in endothelial cells, VSMCs and macrophages [20,22].

Role of the PGs in the vasculature

As in other areas of inflammation, PG generation is increased in human atherosclerotic plaque and the generation of TXA_2 and PGI_2 is increased in patients with atherosclerosis [21]. PGs may contribute to the development of atherosclerosis in several ways. PGs regulate the expression of genes involved in cell growth [23], apoptosis [24] and migration [25], processes that are implicated in the development of atherosclerosis [26]. TXA_2 is a potent activator of platelets, and may therefore contribute to the thrombosis that complicates human atherosclerosis. Platelets release inflammatory proteins such as platelet factor (PF) 4 and CD40L [27,28], in addition to growth factors that are mitogenic for VSMCs. Indeed, TXA_2-receptor antagonists retard plaque formation in hypercholesterolaemic rabbits, prevent arterial thrombosis in rats and decrease atherosclerosis in $apoE^{-/-}$ mice [29–32]. Based on studies with low-dose aspirin, platelet COX-1 appears to be the major source of the increased TXA_2 biosynthesis in atherosclerosis; however, it is possible that other sources may also be involved, as selective COX-1 inhibition fails to completely suppress TXA_2 generation in patients with atherosclerosis [29].

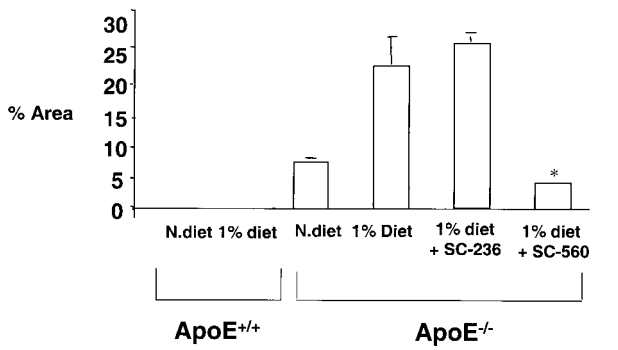
In contrast with TXA_2, in healthy individuals, PGI_2 is formed predominantly by COX-2, presumably in the vascular endothelium [19], whereas both isoforms are responsible for the increase in PGI_2 generation that is seen in patients with atherosclerosis [21]. PGI_2 is a potent platelet inhibitor and vasodilator. PGI_2 also inhibits the proliferation of VSMCs and suppresses foam-cell formation by inhibiting the accumulation of cholesterol esters in macrophages [33]. Thus PGI_2 may influence several steps in the development of atherosclerosis and arterial thrombosis. Indeed, disruption of the PGI_2 receptor (IP) in the low-density lipoprotein (LDL)-receptor mouse shows that PGI_2 has both antithrombotic and anti-inflammatory activities in this model of atherosclerosis [34].

Effect of selective inhibition of COX-1 or COX-2 on the development of atherosclerosis

We have recently examined the effect of COX-1 and COX-2 inhibition on the development of atherosclerosis induced by a diet rich in cholesterol in the $apoE^{-/-}$ mouse. In the present study, we used a selective COX-1 inhibitor, SC-560 [35], and a selective COX-2 inhibitor, SC-236 [36]. Immunohistochemical analysis of cross-sections from the aortic root showed an increase in both COX-1 and COX-2 expression in the atherosclerotic lesions that developed in the aorta. Administration of the selective COX-2 inhibitor, SC-236,

Figure 1 | Effect of selective COX inhibitors on atherosclerotic lesion formation in the apoE$^{-/-}$ mouse

Administration of the COX-2-selective inhibitor, SC-236, had no effect on lesion formation in cholesterol-fed apoE$^{-/-}$ mice. ApoE$^{-/-}$ animals receiving the selective COX-1 inhibitor SC-560 had dramatically decreased lesion formation. Results are means ± S.E.M. where $^{*}P < 0.01$ compared with apoE$^{-/-}$ mice on a high-cholesterol diet.



had no effect on lesion development in this model. However, administration of SC-560, a selective COX-1 inhibitor, dramatically reduced lesion formation (Figure 1). SC-560, however, did not induce regression or prevent the progression of lesions that had already developed (Figure 1), implicating COX-1 in the earlier stages of lesion formation.

PPARs

PPARs are a family of nuclear transcription factors, of which there are four members: α, β, $\gamma 1$ and $\gamma 2$ [37,38] PPARα is expressed mainly in the liver, kidney and brown adipose tissue [39]. It is activated by fibrates, long-chain fatty acids, such as linoleic acid, and eicosanoids, such as LTB_4 (leukotriene B_4) [40,41], and it increases the production of enzymes involved in the β-oxidation of fatty acids [39]. PPARγ is predominantly expressed in adipose tissue where it has a key role in regulating adipocyte differentiation [42]. It is activated by fatty acids, such as CLA (see below) [43], 15-deoxy-$\Delta^{12,14}$-prostaglandin J2 (15d-PGJ2) [44] and several non-steroidal anti-inflammatory drugs (NSAIDs) [45]. The insulin-sensitizing thiazolidinedione drugs also bind with high affinity to PPARγ [46]. When activated by its ligands, PPARγ heterodimerizes with the retinoid X receptor (RXR) and binds to specific peroxisome-proliferator-response elements (PPREs) located in the promoter of target genes [47–49], inducing expression of the target gene.

The structurally distinct and specific PPARγ ligands rosiglitazone and GW7845 were found to inhibit the development of atherosclerosis in the LDL-receptor-deficient mouse [50], which is supported by other studies demonstrating a similar effect with thiazolidinediones in the apoE$^{-/-}$ mouse [51,52]. Several mechanisms may be involved, including an increase in lipid transport in monocyte/macrophages, inhibition of the formation of pro-inflammatory cytokines and suppression of VSMC growth [50,53]. Thus PPARγ agonists have also been reported to inhibit migration of VSMCs and monocytes/macrophages homing to atherosclerotic plaques [54,55].

CLA

The term CLA is used to describe the positional and geometric isomers of linoleic acid. Eight different isomers have been identified, and these differ from each other in the position of the double bonds [56]. CLA is a naturally occurring fatty acid, which is produced in the rumen of ruminant animals by *Butyrovibrio fibrisolvens*, which isomerizes linoleic acid into CLA. CLA may also be synthesized during heat-processing of animal-derived foods. The 9-*cis*, 11-*trans* and the 10-*trans*, 12-*cis* isomers are most predominant in ruminant fats and these account for >80% of total CLA isomers in dairy products [57].

CLA is a naturally occurring PPARγ agonist. CLA was shown to be potentially beneficial in disease when it was found to inhibit chemically induced skin neoplasia in mice [58]. It inhibits tumour growth in a variety of tissues [59,60], and has anti-diabetic effects in rodent models [61]. CLA has been shown to have anti-atherogenic effects [62,63]. Dietary supplementation with 0.5 g of CLA per day for 22 weeks in rabbits fed with an atherogenic diet had fewer fatty lesions in the aorta [62]. Kritchevsky [63] showed that dietary levels of CLA as low as 0.1% in rabbits inhibited atherosclerosis by 34%.

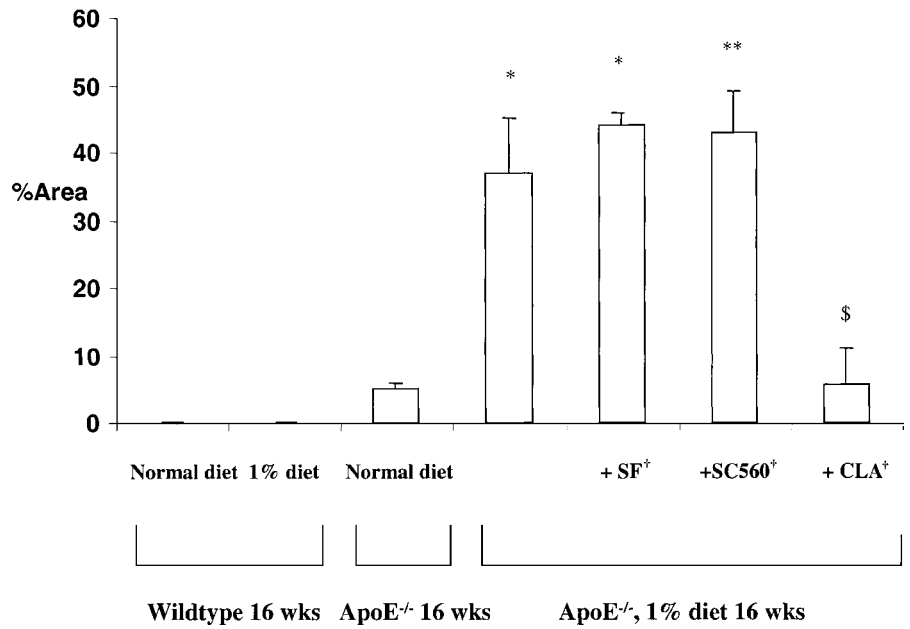
CLA supplementation regresses pre-established atherosclerosis in the apoE$^{-/-}$ mouse model

We have investigated the effect of dietary supplementation of CLA on pre-established atherosclerosis in the apoE$^{-/-}$ mouse model. Administration of 9-*cis*, 11-*trans* CLA with continued administration of a 1% (w/v) cholesterol diet to apoE$^{-/-}$ animals with pre-established atherosclerosis not only retarded further development of atherosclerotic lesions, but also induced regression of the lesion in the aorta (Figure 2).

CLA is a COX inhibitor *in vitro*, yet in our experiments, urinary PG levels, increased in apoE$^{-/-}$ animals receiving the high-cholesterol chow, were unaltered by dietary supplementation with CLA, despite the marked decrease in lesion burden, suggesting that the effects of CLA are independent of COX inhibition. Immunohistochemical analysis of cross-sections from the aortic root showed that expression of PPARγ was increased in animals receiving the CLA supplementation compared with the untreated group, with staining mainly localized to the VSMCs. As previous studies have shown that PPARγ agonists induce the expression of the protein [43], the findings suggest that CLA may be of benefit in atherosclerosis by altering gene expression through activation of PPARγ.

Figure 2 | Effect of CLA supplementation on the progression of pre-established atherosclerosis in the apoE$^{-/-}$ mouse

Administration of the selective COX-1 inhibitor, SC-560, to animals with pre-established atherosclerosis did not prevent the further progression of the disease. Dietary supplementation with CLA did not only prevent further progression of the disease, but also induced regression. Results are means ± S.E.M. †, added after 8 weeks. *, $P < 0.01$ compared with apoE$^{-/-}$ mice on a high-cholesterol diet. **, $P < 0.001$ compared with apoE$^{-/-}$ mice on a high-cholesterol diet with CLA supplementation. $, $P < 0.01$ compared with apoE$^{-/-}$ mice on a high-cholesterol diet with saturated fatty acid supplementation. SF, saturated fat.



Summary

Selective inhibition of COX-1, though not of COX-2, inhibits the development of atherosclerosis coincident with decreased urinary TXB_2 generation in the apoE$^{-/-}$ mouse model. However, inhibition of COX has no effect on the regression of pre-established atherosclerosis. Dietary supplementation with CLA not only prevents progression of pre-established atherosclerosis, but also induces regression. CLA is a natural agonist of the nuclear receptor PPARγ, and, through this mechanism, may have anti-inflammatory activity. Further studies in humans are warranted to examine whether dietary supplementation with CLA may provide a 'nutraceutics' approach to the treatment of atherosclerosis.

References

1 Ross, R. (1999) Am. Heart J. **138**, S419–S420
2 Lusis, A.J. (2000) Nature (London) **407**, 233–241
3 Smith, W.L., Garavito, R.M. and DeWitt, D.L. (1996) J. Biol. Chem. **271**, 33157–33160
4 Garavito, R.M. and DeWitt, D.L. (1999) Biochim. Biophys. Acta **1441**, 278–287
5 Tazawa, R., Xu, X.M., Wu, K.K. and Wang, L.H. (1994) Biochem. Biophys. Res. Commun. **203**, 190–199
6 Wu, K.K. (1997) Adv. Exp. Med. Biol. **400A**, 121–126
7 Smith, W.L., DeWitt, D.L. and Garavito, R.M. (2000) Annu. Rev. Biochem. **69**, 145–182
8 Meade, E.A., McIntyre, T.M., Zimmerman, G.A. and Prescott, S.M. (1999) J. Biol. Chem. **274**, 8328–8334
9 Smith, W.L. (1989) Biochem. J. **259**, 315–324
10 McAdam, B.F., Mardini, I.A., Habib, A., Burke, A., Lawson, J.A., Kapoo, R.S. and FitzGerald, G.A. (2000) J. Clin. Invest. **105**, 1473–1482
11 Jones, D.A., Carlton, D.P., McIntyre, T.M., Zimmerman, G.A. and Prescott, S.M. (1993) J. Biol. Chem. **268**, 9049–9054
12 Xie, W. and Herschman, H.R. (1996) J. Biol. Chem. **271**, 31742–31748
13 Schmedtje, Jr, J.F., Ji, Y.S., Liu, W.L., DuBois, R.N. and Runge, M.S. (1997) J. Biol. Chem. **272**, 601–608
14 Adderley, S. and Fitzgerald, D.J. (1999) J. Biol. Chem. **274**, 5038–5046
15 Hoffmann, C. (2000) Curr. Med. Chem. **7**, 1113–1120
16 Harris, R.C., McKanna, J.A., Akai, Y., Jacobson, H.R., Dubois, R.N. and Breyer, M.D. (1994) J. Clin. Invest. **94**, 2504–2510
17 Morham, S.G., Langenbach, R., Loftin, C.D., Tiano, H.F., Vouloumanos, N., Jennette, J.C., Mahler, J.F., Kluckman, K.D., Ledford, A., Lee, C.A. et al. (1995) Cell **83**, 473–482
18 Loftin, C.D., Trivedi, D.B., Tiano, H.F., Clark, J.A., Lee, C.A., Epstein, J.A., Morham, S.G., Breyer, M.D., Nguyen, M., Hawkins, B.M. et al. (2001) Proc. Natl. Acad. Sci. U.S.A. **98**, 1059–1064
19 Cullen, L., Kelly, L., Connor, S.O. and Fitzgerald, D.J. (1998) J. Pharmacol. Exp. Ther. **287**, 578–582
20 Schonbeck, U., Sukhova, G.K., Graber, P., Coulter, S. and Libby, P. (1999) Am. J. Pathol. **155**, 1281–1291
21 Belton, O., Byrne, D., Kearney, D., Leahy, A. and Fitzgerald, D.J. (2000) Circulation **102**, 840–845
22 Baker, C.S., Hall, R.J., Evans, T.J., Pomerance, A., Maclouf, J., Creminon, C., Yacoub, M.H. and Polak, J.M. (1999) Arterioscler. Thromb. Vasc. Biol. **19**, 646–655
23 Bornfeldt, K.E., Campbell, J.S., Koyama, H., Argast, G.M., Leslie, C.C., Raines, E.W., Krebs, E.G. and Ross, R. (1997) J. Clin. Invest. **100**, 875–885
24 Brown, M.R., Miller, F.J.J., Li, W.G., Ellingson, A.N., Mozena, J.D., Chatterjee, P., Engelhard, T.J.F., Zwacka, R.M., Oberley, L.W., Fang, X. et al. (1999) Circ. Res. **85**, 524–533
25 Attiga, F.A., Fernandez, P.M., Weeraratna, A.T., Manyak, M.J. and Patierno, S.R. (2000) Cancer Res. **60**, 4629–4637
26 Ross, R. (1993) Nature (London) **362**, 801–809
27 Andre, P., Nannizzi-Alaimo, L., Prasad, S.K. and Phillips, D.R. (2002) Circulation **106**, 896–899
28 Park, S.M., Jung, H.Y., Kim, H.O., Rhim, H., Paik, S.R., Chung, K.C., Park, J.H. and Kim, J. (2002) Blood **100**, 2506–2514

29 Belton, O. and Fitzgerald, D. (2003) J. Am. Coll. Cardiol. **41**, 1820–1822
30 Osborne, J.A. and Lefer, A.M. (1988) Am. J. Physiol. **255**, H318–H324
31 Tanaka, T., Sato, R. and Kurimoto, T. (2000) Eur. J. Pharmacol. **401**, 413–418
32 Cayatte, A.J., Du, Y., Oliver-Krasinski, J., Lavielle, G., Verbeuren, T.J. and Cohen, R.A. (2000) Arterioscler. Thromb. Vasc. Biol. **20**, 1724–1728
33 Willis, A.L., Smith, D.L., Vigo, C. and Kluge, A.F. (1986) Lancet **2**, 682–683
34 Murata, T., Ushikubi, F., Matsuoka, T., Hirata, M., Yamasaki, A., Sugimoto, Y., Ichikawa, A., Aze, Y., Tanaka, T., Yoshida, N. et al. (1997) Nature (London) **388**, 678–682
35 Smith, C.J., Zhang, Y., Koboldt, C.M., Muhammad, J., Zweifel, B.S., Shaffer, A., Talley, J.J., Masferrer, J.L., Seibert, K. and Isakson, P.C. (1998) Proc. Natl. Acad. Sci. U.S.A. **95**, 13313–13318
36 Penning, T.D., Talley, J.J., Bertenshaw, S.R., Carter, J.S., Collins, P.W., Docter, S., Graneto, M.J., Lee, L.F., Malecha, J.W., Miyashiro, J.M. et al. (1997) J. Med. Chem. **40**, 1347–1365
37 Barbier, O., Torra, I.P., Duguay, Y., Blanquart, C., Fruchart, J.C., Glineur, C. and Staels, B. (2002) Arterioscler. Thromb. Vasc. Biol. **22**, 717–726
38 Kliewer, S.A., Xu, H.E., Lambert, M.H. and Willson, T.M. (2001) Recent Prog. Horm. Res. **56**, 239–263
39 Braissant, O., Foufelle, F., Scotto, C., Dauca, M. and Wahli, W. (1996) Endocrinology **137**, 354–366
40 Couturier, C., Brouillet, A., Couriaud, C., Koumanov, K., Bereziat, G. and Andreani, M. (1999) J. Biol. Chem. **274**, 23085–23093
41 Moya-Camarena, S.Y. and Belury, M.A. (1999) Nutr. Rev. **57**, 336–340
42 Spiegelman, B.M. (1998) Diabetes **47**, 507–514
43 Yu, Y., Correll, P.H. and Vanden Heuvel, J.P. (2002) Biochim. Biophys. Acta **1581**, 89–99
44 Forman, B.M., Tontonoz, P., Chen, J., Brun, R.P., Spiegelman, B.M. and Evans, R.M. (1995) Cell **83**, 803–812
45 Kliewer, S.A., Sundseth, S.S., Jones, S.A., Brown, P.J., Wisely, G.B., Koble, C.S., Devchand, P., Wahli, W., Willson, T.M., Lenhard, J.M. and Lehmann, J.M. (1997) Proc. Natl. Acad. Sci. U.S.A. **94**, 4318–4323
46 Lehmann, J.M., Moore, L.B., Smith-Oliver, T.A., Wilkison, W.O., Willson, T.M. and Kliewer, S.A. (1995) J. Biol. Chem. **270**, 12953–12956
47 Chinetti, G., Fruchart, J.C. and Staels, B. (2001) Z. Kardiol. **90**, Suppl. 3, 125–132
48 Ziouzenkova, O., Perrey, S., Marx, N., Bacqueville, D. and Plutzky, J. (2002) Curr. Atheroscler. Rep. **4**, 59–64
49 Ricote, M., Huang, J.T., Welch, J.S. and Glass, C.K. (1999) J. Leukoc. Biol. **66**, 733–739
50 Kersten, S., Desvergne, B. and Wahli, W. (2000) Nature (London) **405**, 421–424
51 Chen, Z., Ishibashi, S., Perrey, S., Osuga, J., Gotoda, T., Kitamine, T., Tamura, Y., Okazaki, H., Yahagi, N., Iizuka, Y. et al. (2001) Arterioscler. Thromb. Vasc. Biol. **21**, 372–377
52 Collins, A.R., Meehan, W.P., Kintscher, U., Jackson, S., Wakino, S., Noh, G., Palinski, W., Hsueh, W.A. and Law, R.E. (2001) Arterioscler. Thromb. Vasc. Biol. **21**, 365–371
53 Fukunaga, Y., Itoh, H., Doi, K., Tanaka, T., Yamashita, J., Chun, T.H., Inoue, M., Masatsugu, K., Sawada, N., Saito, T. et al. (2001) Atherosclerosis **158**, 113–119
54 Pasceri, V., Wu, H.D., Willerson, J.T. and Yeh, E.T. (2000) Circulation **101**, 235–238
55 Marx, N., Sukhova, G., Murphy, C., Libby, P. and Plutzky, J. (1998) Am. J. Pathol. **153**, 17–23
56 Eulitz, K., Yurawecz, M.P., Sehat, N., Fritsche, J., Roach, J.A., Mossoba, M.M., Kramer, J.K., Adlof, R.O. and Ku, Y. (1999) Lipids **34**, 873–877
57 Chin, S.F., Storkson, J.M., Liu, W., Albright, K.J. and Pariza, M.W. (1994) J. Nutr. **124**, 694–701
58 Ha, Y.L., Grimm, N.K. and Pariza, M.W. (1987) Carcinogenesis **8**, 1881–1887
59 Fu, Y., Luo, N. and Lopes-Virella, M.F. (2002) Atherosclerosis **160**, 11–20
60 Belury, M.A. (1995) Nutr. Rev. **53**, 83–89
61 Anon. (1998) Biochem. Biophys. Res. Commun. **247**, 911
62 Lee, K.N., Kritchevsky, D. and Pariza, M.W. (1994) Atherosclerosis **108**, 19–25
63 Kritchevsky, D. (2000) Br. J. Nutr. **83**, 459–465

Received 14 July 2003