Invited commentary

Good COP, bad COP: an unsolved murder. Are dietary cholesterol oxidation products guilty of atherogenicity?

The link between elevated plasma cholesterol concentrations and atherosclerosis is well established, but the role of cholesterol oxidation products (COP), also termed oxysterols, is still controversial. Relevant, extensive reviews include those of Brown & Jessup (1999), Schroepfer (2000), Björkhem & Diczfalusy (2002) and Garcia-Cruset *et al.* (2002). Oxysterols appear to be atherogenic in some, but not all, studies in animal models; *in vitro*, oxysterols exhibit various effects, many of which are potentially pro-atherogenic, including toxicity to macrophages, smooth muscle cells and endothelial cells (for reviews, see Brown & Jessup, 1999; Schroepfer, 2000; Björkhem & Diczfalusy, 2002; Garcia-Cruset *et al.* 2002). Death of macrophage foam cells leads to the development of the lipid core of advanced atherosclerotic lesions whilst death of smooth muscle cells thins the fibrous cap, and these changes destabilise lesions, predisposing them to rupture with thrombogenic consequences. Impairment of endothelial cells leads to loss of barrier function and promotes cell adhesion and coagulation. The results of a new study, published in the present issue of the *British Journal of Nutrition* (Ando *et al.* 2002), in an animal model provides evidence that dietary oxysterols are non-atherogenic.

Oxysterols usually occur at low levels accompanied by high concentrations of the parent cholesterol. Oxysterols have long been known to occur in samples of cholesterol that have been exposed to oxygen from the air as a result of prolonged storage or by heating. There are many reports of oxysterols in human atherosclerotic lesions (Brown & Jessup, 1999). Six oxysterols identified in human advanced lesions totalled 18 µg/mg cholesterol (sum of average levels of each individual oxysterol), whereas in macroscopically normal specimens of artery distant from lesions, oxysterols were about 10-fold less abundant (Garcia-Cruset *et al.* 2001). In macroscopically normal artery from lesion margins, oxysterol levels were between these two extremes (KLH Carpenter and IR Challis, unpublished results). Oxysterol levels (standardised for cholesterol) reported in normal human plasma (Babiker & Diczfalusy, 1998) are about 100-fold lower than in human advanced atherosclerotic lesions. In smokers, plasma levels of 7-ketocholesterol and of α-epoxycholesterol were 40 and 50 % higher respectively than in non-smoker control subjects (Mol *et al.* 1997). Human plasma levels of 7β-hydroxycholesterol were associated with progression of carotid atherosclerosis (Salonen *et al.* 1997). Long-term vitamin E supplementation

reduced human plasma levels of 7β-hydroxycholesterol (Porkkala-Sarataho *et al.* 2000).

Relatively high levels of oxysterols occur in certain foods, notably meat, egg and dairy products that have been subjected to heating in the presence of air and/or to prolonged storage in air, e.g. spray-dried powdered milk and egg, and ghee (clarified butter). Consumption of ghee (123 mg oxysterols/g total sterols) was suggested as a factor in the elevated morbidity and mortality from atherosclerosis in Indian immigrants in London, UK, compared with indigenous Londoners (Jacobson, 1987).

In atherosclerotic lesions, to what extent are oxysterols derived from the diet or produced *in vivo*? Moreover, how much is produced *in situ* in the lesion itself? For the 7-oxysterols, the epoxy-cholesterols and cholestanetriol, these questions remain unanswered. 27-Hydroxycholesterol (also termed 26-hydroxycholesterol in some of the literature) is not an auto-oxidation product of cholesterol, but is produced by cytochrome P450 sterol 27-hydroxylase, a mitochondrial enzyme in the liver (as part of the bile acid synthesis pathway) and in extra-hepatic tissues (Shanahan *et al.* 2001; Björkhem & Diczfalusy, 2002). This enzyme occurs in macrophages in human advanced atherosclerotic lesions (Crisby *et al.* 1997; Shanahan *et al.* 2001). 27-Hydroxycholesterol levels in lesions increase with lesion severity (Carpenter *et al.* 1995; Garcia-Cruset *et al.* 2001). In advanced lesions, 27-hydroxycholesterol is the most abundant oxysterol (Garcia-Cruset *et al.* 2001) and it is more abundant in the lipid core than in the fibrous cap (Garcia-Cruset *et al.* 1999). 27-Hydroxycholesterol is also the most abundant oxysterol in human plasma (Babiker & Diczfalusy, 1998).

27-Hydroxylase is believed to constitute a cholesterol removal mechanism from extra-hepatic cells. 27-Hydroxycholesterol leaves cells more readily than the parent cholesterol and, moreover, 27-hydroxylase can oxidise 27-hydroxycholesterol further to form 3β-hydroxycholestenoic acid, which exits even more readily (Babiker *et al.* 1999). There is a flux of 27-hydroxycholesterol and 3β-hydroxycholestenoic acid from the tissues to the liver, where these compounds are converted to bile acids (Björkhem & Diczfalusy, 2002). 27-Hydroxylase can also act on 7-oxysterols as substrates, preventing macrophages accumulating 7-ketocholesterol (Brown *et al.* 2000), which might constitute an oxysterol-removal mechanism (analogous to that for cholesterol) from atherosclerotic lesions. Plasma oxysterols are associated with lipoproteins,

apart from 3β-hydroxycholestenoic acid, which is in the lipoprotein-free fraction (Björkhem & Diczfalusy, 2002).

Many oxysterols can be produced non-enzymatically, as illustrated by Ando *et al.* (2002), including 7α- and 7β-hydroxycholesterols, 5α,6α- and 5β,6β-epoxycholesterols, cholestanetriol, 7-ketocholesterol (also termed 7-oxocholesterol) and 25-hydroxycholesterol. 7α-Hydroxycholesterol can also be produced enzymatically by hepatic 7α-hydroxylase. Plasma 7α-hydroxycholesterol is at least partly derived from 'leakage' from the liver (Björkhem & Diczfalusy, 2002). 27-Hydroxycholesterol is a purely enzymatic product (see earlier). Recently, a sterol 25-hydroxylase that produces 25-hydroxycholesterol has been characterised (Lund *et al.* 1998). 7α- and 7β-Hydroxycholesterols and 7-ketocholesterol can arise from decomposition of 7α- and 7β-hydroperoxycholesterols, which are produced by free radical oxidation of cholesterol. 7β-Hydroxycholesterol is usually more abundant than its 7α-isomer because the former is less sterically hindered. Cholestanetriol is believed to arise from opening of the epoxide ring of 5,6-epoxycholesterols.

The 7-position of cholesterol is vulnerable to free radical attack because abstraction of a H atom from this position produces an allylic radical, i.e. the unpaired electron is resonance-stabilised by the C–C double bond of the cholesterol molecule. Oxidation of cholesterol is markedly promoted by the presence of polyunsaturated fatty acids, where peroxidation of the latter (either non-enzymatically or by the action of lipoxygenase) gives rise to polyunsaturated fatty acid-derived radicals that proceed to attack cholesterol at the 7-position. This occurs in oxidation of LDL *in vitro* by macrophages or by Cu^{2+} ions (Carpenter *et al.* 1994). Oxidised LDL has been detected in atherosclerotic lesions both in human subjects and apolipoprotein (Apo) E-deficient mice (Pratico, 2001).

There have been many attempts to model human atherosclerosis using animals. Imai *et al.* (1976) produced evidence that the atherogenicity and angiotoxicity of dietary cholesterol to rabbits was due to oxysterols present as auto-oxidation impurities in the cholesterol used to supplement the diets. Numerous studies have investigated the atherogenicity of dietary oxysterols in animals (see reviews mentioned earlier), but the overall effect of oxysterols was unclear, some studies suggesting an atherogenic effect, whilst others showed no effect or even an anti-atherogenic effect.

Recently, dietary oxysterols were shown to be atherogenic by Staprans *et al.* (2000) in a strain of mice genetically engineered to be deficient in ApoE, an animal model in which plasma cholesterol levels are elevated due to the accumulation of VLDL and chylomicron remnants. In mice consuming the oxysterol-supplemented diet, oxysterol levels increased in plasma, but no measurements were made of oxysterols in the artery (aorta) wall.

The new study in ApoE-deficient mice (Ando *et al.* 2002) assessed the lesion volume (as lesion area in transverse sections) in the aortic root, as well as levels of cholesterol and oxysterols in serum, liver and aorta, resulting from diets supplemented with cholesterol (200 mg/kg diet), or with oxysterols (200 mg/kg diet) produced by

heating cholesterol, or without supplements (control). Neither the cholesterol-supplemented diet nor the oxysterol-supplemented diet resulted in significant change in size of lesions or aortic cholesterol concentration compared with the control diet. Except for 27-hydroxycholesterol, levels of oxysterols increased significantly in the serum and livers of mice supplemented with dietary oxysterols, though the percentage increase in liver oxysterol levels was less dramatic than in serum. In the aortas, by contrast, only 7-ketocholesterol and cholestanetriol increased significantly, but not as markedly as in serum. Setting aside 27-hydroxycholesterol (an entirely enzymatic COP, absent from the dietary supplement of oxysterols), the ranking order of relative abundances of the individual oxysterols was different in diet, serum, liver and aorta. Oxysterols are thus not simply taken up *en masse* from diet to serum to tissues, and the profile of the dietary oxysterols is modulated *in vivo*, almost certainly involving differential uptake, synthesis, metabolism and removal. Oxysterols formed *in vivo* are 7α- and 7β-hydroxycholesterols, 7-ketocholesterol, and 24-, 25- and 27-hydroxycholesterols, demonstrated in rats by $^{18}O_2$ inhalation (Breuer & Björkhem, 1995).

Surprisingly, among the results of Ando *et al.* (2002) is the apparent lack of aortic uptake of oxysterols, apart from 7-ketocholesterol and cholestanetriol, which might both be formed at least partly *in situ* in the artery wall. It is often tacitly assumed in biological studies that plasma (or serum) and tissue levels of a substance will reflect each other to some extent, and the study of Ando *et al.* (2002) strikingly illustrates that this is not always true. Another such example is in the human disease cerebrotendinous xanthomatosis, in which plasma cholesterol levels are usually normal, but tissue levels of cholesterol are abnormally high due to a genetic deficiency of sterol 27-hydroxylase (a cholesterol removal mechanism; see earlier); cerebrotendinous xanthomatosis sufferers often develop premature atherosclerosis (Björkhem & Diczfalusy, 2002).

The reason for the apparent discrepancy between the findings of Ando *et al.* (2002) and Staprans *et al.* (2000), i.e. dietary oxysterols were non-atherogenic in the former but atherogenic in the latter, is unknown. Whilst there were some differences in conditions between the two studies, the levels of oxysterols in the diets were approximately the same, and so were the serum cholesterol concentrations. The study by Staprans *et al.* (2000) involved a 4-month dietary supplementation period whereas that of Ando *et al.* (2002) was 8 weeks. Another possible important difference is as follows. Ando *et al.* (2002) oxidised cholesterol then used column chromatography (silica gel) to remove most of the residual cholesterol and isolate an oxysterol fraction that was used to supplement the diet. Staprans *et al.* (2000), in contrast, did not fractionate the oxidised cholesterol, so that the oxysterols in the diet were accompanied by unoxidised parent cholesterol. Dietary cholesterol might act as a 'vehicle' for dietary oxysterols, as serum levels of oxysterols, recalculated as μg/mg cholesterol for the study of Ando *et al.* (2002), appeared somewhat lower than those of Staprans *et al.* (2000). Moreover, some uncharacterised pro-atherogenic species might

have been lost in the fractionation process of Ando *et al.* (2002).

ApoE-deficient mice have become widely used over the past decade to model atherosclerosis (Breslow, 1996; Moghadasian *et al.* 2001). ApoE normally mediates uptake of remnant lipoproteins by several receptor systems in the liver. If ApoE is deficient or defective, VLDL and chylomicron remnants accumulate in the plasma due to poor clearance, and the plasma cholesterol concentration is markedly elevated. VLDL, chylomicrons and their remnants are triacylglycerol-rich lipoproteins, normally containing ApoE, and they also contain cholesterol, cholesteryl ester and phospholipid. The VLDL that accumulates if ApoE is deficient or defective is termed β-VLDL, containing higher levels of cholesteryl ester than normal VLDL. In man, this condition is termed type III hyperlipidaemia and results in premature atherosclerosis. The lesions of ApoE-deficient mice range from early (fatty streak) to advanced (fibrous plaque), and dietary supplementation with fat, cholesterol and cholic acid accelerates lesion progression. Lipid-rich, unstable advanced lesions with thinned fibrous caps were reported in ApoE-deficient mice aged 17 months (Moghadasian *et al.* 2001), i.e. older than the mice in the studies of Ando *et al.* (2002) and Staprans *et al.* (2000), where the lesions were at an earlier stage, described as fatty streaks by Staprans *et al.* (2000). ApoE is produced by the liver and it is also secreted by macrophages. Bone-marrow transplantation from normal mice into ApoE-deficient mice diminished the progression of atherosclerosis and reduced serum cholesterol levels (Van Eck *et al.* 2000).

Oxidative stress appears to be involved in atherosclerosis in ApoE-deficient mice and in human subjects (Hayek *et al.* 1994; Pratico, 2001). Recently, Rosenblat & Aviram (2002) produced evidence for oxysterol-induced activation of NADPH-oxidase in ApoE-deficient mouse macrophages, enhancing LDL oxidation *in vitro*. Dietary supplementation with natural antioxidants (vitamin E or vitamin E plus ubiquinone) was anti-atherogenic in ApoE-deficient mice (Pratico *et al.* 1998; Thomas *et al.* 2001). In the latter study, the antioxidant supplementation lowered aortic levels of lipid hydroperoxides and 7-ketocholesterol when expressed per mg protein, but when standardised for cholesterol, the lowering of 7-ketocholesterol was not statistically significant. Ando *et al.* (2002) noted that all the diets used in their study contained the artificial antioxidant *tert*-butylhydroquinone, which might have counteracted the potential pro-oxidant, pro-atherogenic effects of the oxysterols.

Oxysterols can act as regulators of cholesterol homeostasis in various ways (Brown & Jessup, 1999; Björkhem & Diczfalusy, 2002), which might counteract the potential atherogenic effects of the oxysterols. The down-regulation by oxysterols of hydroxymethyl glutaryl CoA reductase (a key enzyme in cholesterol biosynthesis) is among these. In addition, some oxysterols are activating ligands for liver X receptors, transcription factors regulating several genes important in cholesterol homeostasis, e.g. the ABCA1 cholesterol transporter pathway, resulting in efflux of cholesterol from macrophages. Oxysterols themselves might be similarly exported along with cholesterol, constituting a self-limiting mechanism for oxysterol levels in lesions. Furthermore, the toxicity of several of the oxysterols to macrophages *in vitro* was inhibited by cholesterol (Clare *et al.* 1995). Such an effect, if true *in vivo*, where cholesterol is abundant, might at least partly explain the lack of atherogenicity of dietary oxysterols in the study of Ando *et al.* (2002). Serum oxysterol levels in human subjects are considerably lower (whether expressed per litre serum or per mg cholesterol) than those achieved in ApoE-deficient mice receiving dietary oxysterol supplementation (Staprans *et al.* 2000; Ando *et al.* 2002).

The results of Ando *et al.* (2002) will be reassuring to some, as providing evidence for the defence of dietary oxysterols, alias COP, on this occasion acquitting them of promoting atherosclerosis in ApoE-deficient mice under the conditions of their study. As to whether dietary oxysterols are atherogenic in man, the jury is still out.

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