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Effect of Circulating Forms of Soy Isoflavones on the Oxidation of Low Density Lipoprotein

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Soy isoflavones are thought to have a cardioprotective effect that is partly mediated by an inhibitory influence on the oxidation of low density lipoprotein (LDL). However, the aglycone forms investigated in many previous studies do not circulate in appreciable quantities because they are metabolised in the gut and liver. We investigated effects of various isoflavone metabolites, including for the first time the sulphated conjugates formed in the liver and the mucosa of the small intestine, on copper-induced LDL oxidation. The parent aglycones inhibited oxidation, although only 5% as well as quercetin. Metabolism increased or decreased their effectiveness. Equol inhibited 2.65-fold better than its parent compound daidzein and 8-hydroxydaidzein, not previously assessed, was 12.5-fold better than daidzein. However, monosulphated conjugates of genistein, daidzein and equol were much less effective and disulphates completely ineffective. Since almost all isoflavones circulate as conjugates, these data suggest that despite the increased potency produced by some metabolic changes, isoflavones may not be effective antioxidants *in vivo* unless they are deconjugated again.

Keywords: Soy; Isoflavones; Antioxidant; Low density lipoprotein; Equol; 8-Hydroxydaidzein

INTRODUCTION

There is increasing evidence that dietary soy has health promoting effects, particularly in relation to the chronic diseases, such as coronary heart disease, that account for the majority of morbidity and mortality in many industrialised countries. Indeed, the US Food and Drug Administration have approved a health claim for the cardioprotective influence of soy. This approval is based on its cholesterol-lowering effect,[1] but soy has other properties that may slow the development of atherosclerosis and its sequelae. Much recent attention has focused on the isoflavones of soy, which are known to have antioxidant[2] and oestrogenic[3] properties and to exert other potentially beneficial effects on cells.[4,5]

The principal isoflavones in soy are the glucosides, acetyl-glucosides and malonyl-glucosides of the aglycones, genistein and daidzein. These compounds are hydrolysed to genistein and daidzein by β-glycosidases in the gut, which allows them to be transported across the gut wall;[6–8] studies of potential anti-atherogenic properties have therefore concentrated on these aglycones. However, they constitute only a small fraction of the soy isoflavone derivatives circulating in blood because they are subject to further metabolism.[9,10] In the gut, they can be converted by bacterial enzymes first to dihydrogenistein and dihydrodaidzein, and then to 6′-hydroxy-*O*-desmethylangolensin and *O*-desmethylangolensin (*O*-DMA); dihydrodaidzein can also be metabolised to equol.[10–12] All these compounds can be absorbed and are modified further within the mucosa of the small intestine and by the liver. First, the majority of the aglycones are conjugated with glucuronic acid or sulphuric acid.[13–16] Both the mono- and di-conjugates

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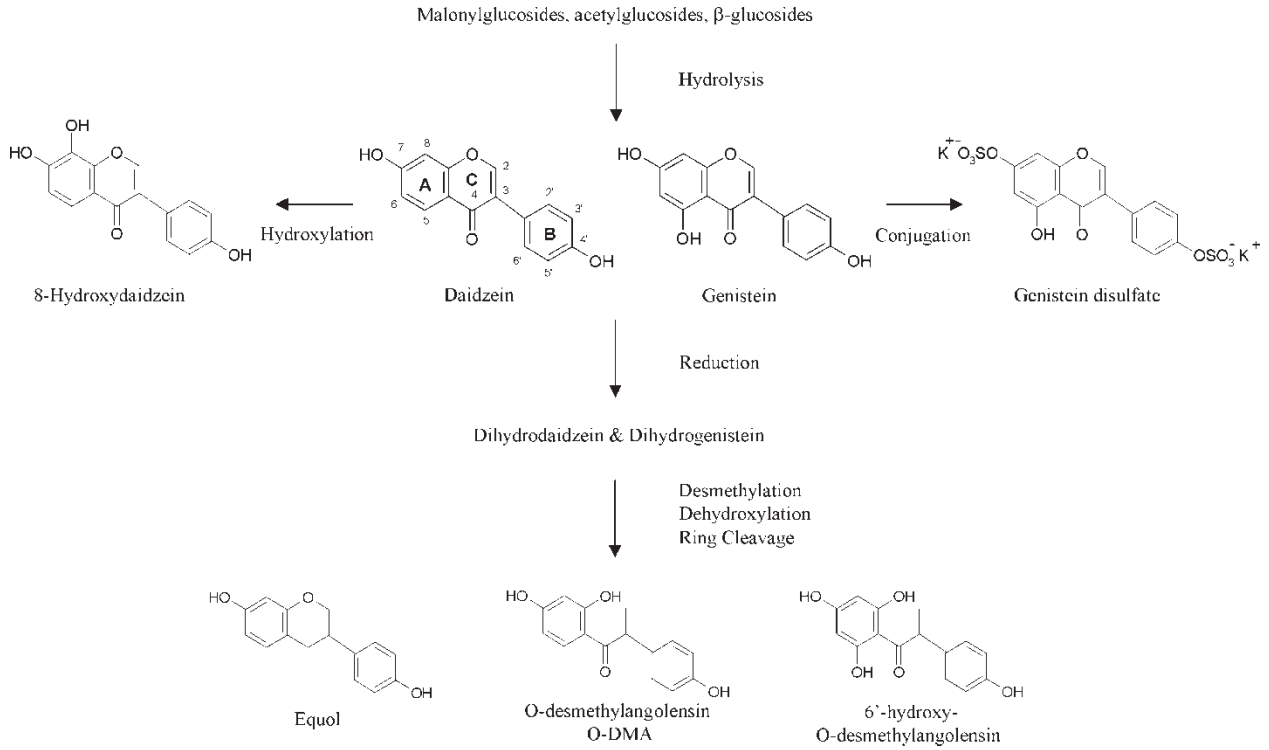

FIGURE 1 Structures of the isoflavone aglycones used in this study and selected metabolites, with a diagrammatic indication of the metabolic pathways.

of each type, and the sulphoglucuronides, can be formed. Second, mono- and di-hydroxylated products of genistein and daidzein are produced.[17] A major daidzein metabolite of this type is 8-hydroxydaidzein (8-OH-daidzein). Structures are given in Fig. 1.

Clearly it is the antiatherogenic properties of these compounds, rather than the parent aglycones, that should be assessed. The complexity and diversity of the metabolic pathways means that a large number of different compounds could be investigated. In the present work, the parent aglycones, examined in previous studies, have been compared with equol, with the mono- and di-sulphated conjugates of all three, with *O*-DMA and with 8-OH-daidzein. Their influence on the oxidation of low density lipoprotein (LDL) was determined, as an example of their potential anti-atherogenic properties. Quercetin, a dietary flavonoid that is known to strongly influence such oxidation,[18] was used as a positive control.

METHODS

Test Compounds

Quercetin, genistein and daidzein were obtained from Sigma. Equol, *O*-DMA and 8-OH-daidzein were obtained from Plantech Ltd (Reading, UK). Genistein, daidzein and equol sulphates have recently been synthesised by Drs B. Fairley, N. Botting, and J. Coley (MRC Dunn Human Nutrition Unit, Cambridge)[19] and were made available to us.

LDL Isolation

Blood from healthy male volunteers aged 20–30 years was collected into EDTA-coated vacutainers under sterile conditions. Informed consent was obtained and the study received ethical approval from the University of Reading. Plasma was prepared by low speed centrifugation and LDL was isolated from it by standard methods of isopycnic ultracentrifugation. Briefly, the plasma was adjusted to a density of 1.21 g/ml with KBr, overlain with a 1.296 g/ml solution and spun at 200,000*g* for 1 h. The LDL-containing band was adjusted with KBr to a density of 1.063 g/ml, overlain with a 1.006 g/ml solution and spun at 200,000*g* for 3 h. The density solutions contained EDTA and all spins were carried out at 4°C.

The LDL was exhaustively dialysed against low-phosphate, EDTA-free "dialysis buffer" (140 mmol/l NaCl, 8.1 mmol/l Na_2HPO_4, 1.9 mmol/l NaH_2PO_4, pH 7.4) before use, and its protein concentration was determined by the modified Lowry assay of Schacterle and Pollack[20] using bovine serum albumin as a standard.

Oxidation Experiments

Oxidation of LDL was induced by copper and was assessed from the absorbance at 234 nm, which

indicates the concentration of conjugated dienes, or from electrophoretic mobility on agarose gels, which indicates the net charge on the molecule.

Spectrophotometric Measurements and their Analysis

Test solutions, each with a volume of 3 ml, were either

(i) Samples—LDL (50 μg protein/ml), $CuSO_4$ (5 μmol/l), and test compound (isoflavone, isoflavone metabolite or quercetin) (≤5 μmol/l),
(ii) References—LDL (50 μg protein/ml) and $CuSO_4$ (5 μmol/l), but no test compound, or
(iii) Blanks—LDL (50 μg protein/ml) and test compound (≤5 μmol/l), but no $CuSO_4$ and hence no induced oxidation.

Isoflavones and their metabolites were initially dissolved in DMSO but were then diluted in dialysis buffer so that there was never more than 0.01% DMSO present in the final test solution. $CuSO_4$ was dissolved in dialysis buffer. All solutions were maintained at 37°C in quartz cuvettes in the spectrophotometer (Lambda Bio40 dual beam, Perkin-Elmer). Absorbance was monitored continuously, the addition of copper defining the start of each experiment. Absorbances obtained for the blanks were subtracted from sample and reference values.

Idealised Esterbauer kinetics[21] imply no increase in absorbance during the lag phase and a nearly linear increase during the propagation phase. In practice, absorbance values show a more sigmoid trend with time. Consequently, the end of the lag phase was defined by the intersection of two lines: the first was obtained by linear extrapolation of absorbances in the initial part of the lag phase and the second was the tangent to the steepest part of the absorbance curve during the propagation phase. Both lines were fitted by least-squares regression. The duration of the lag phase was calculated for LDL in the absence and presence of test compounds, and any increase in duration caused by the test compounds was expressed as a percentage. Experiments were conducted in triplicate. Data are presented as mean ± SEM.

Electrophoretic Measurements

Two test compounds (8-OH-daidzein and equol disulphate) were used; they were chosen because the former gave the largest increase in lag phase and the latter, despite being a metabolite of the same parent compound, had a negligible effect (see below). Test solutions, each with a volume of 200 μl, were initially either:

(i) Samples—LDL (50 μg protein/ml), $CuSO_4$ (5 μmol/l), and test compound (8-OH-daidzein or equol disulphate) (5 μmol/l),
(ii) References—LDL (50 μg protein/ml) and $CuSO_4$ (5 μmol/l), but no test compound, or
(iii) Controls—LDL (50 μg protein/ml) on its own or with 8-OH-daidzein (5 μmol/l) and EDTA (5 μmol/l), but no $CuSO_4$ and hence no induced oxidation.

In subsequent experiments, these concentrations (also used in the lag phase assay) were increased 10-fold, in order to increase the visibility of bands on the gels. As in the lag phase assay, the isoflavones and their metabolites were initially dissolved in DMSO but were then diluted in dialysis buffer so that there was never more than 0.01% DMSO present in the final test solution, the $CuSO_4$ was dissolved in dialysis buffer, and all solutions were maintained at 37°C. The start of the experiment was defined by the addition of the copper, and its termination by the addition of EDTA. Durations of 3 h (2 experiments), 4 h (1 experiment), 5 h (3 experiments) and 6 h (2 experiments) were used; all but two of the experiments were conducted in duplicate.

The electrophoretic shift assay was performed using the Paragon® system (Beckman Coulter, USA). Samples were applied to Lipo gels (0.5% agarose) and electrophoresed at 100 V for 30 min, using baribital buffer (0.05 mol/l, pH = 8.6). Gels were fixed for 5 min using a mixture of ethanol, deionised water and glacial acetic acid (6:3:1 by volume). They were then dried for 2 h, stained with Paragon® lipoprotein stain for 5 min, washed using 45% (vol/vol) ethanol, and dried again for 2 h.

RESULTS

The effects of the parent aglycones, their unconjugated gut and liver metabolites, and quercetin on the duration of the lag phase are shown in Fig. 2; compounds are ordered according to the size of their effect. At the highest concentration (5 μmol/l), genistein and daidzein increased the duration of the lag phase by around 50% compared to the samples with no added flavonoids. The gut metabolite *O*-DMA had a slightly smaller effect. The remaining compounds were much more effective: equol doubled the lag phase duration, 8-OH-daidzein increased it 5-fold, and quercetin increased it by an order of magnitude.

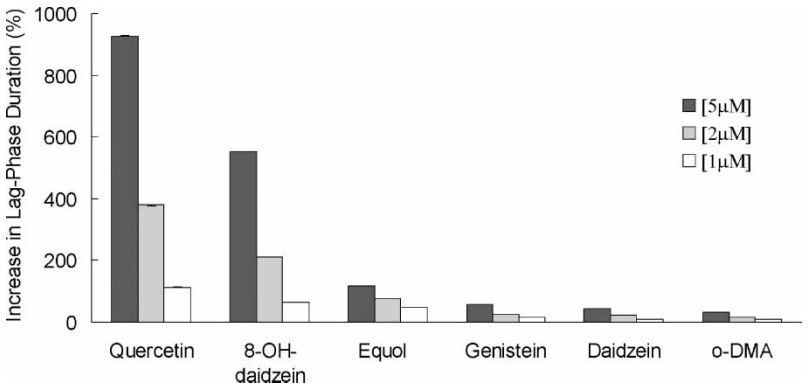

FIGURE 2 Effects of (i) quercetin, (ii) genistein and daidzein (the aglycone forms of the major soy isoflavones), and (iii) their metabolites equol, 8-OH-daidzein and *O*-DMA, at concentrations of 1, 2 and 5 μmol/l, on the duration of the lag phase during oxidation of LDL by copper. Bars show means ± SEM's of triplicate experiments. (SEM's are too small to see in many cases.)

For all compounds, Fig. 2 shows a smooth dependence of antioxidant effect on concentration. For clarity, only results for 1, 2 and 5 μmol/l are shown; however, lower concentrations were studied. As an example of the latter data, Fig. 3 shows all concentrations examined for 8-OH-daidzein. There was a consistent trend with concentration, and effects were seen even at concentrations below 1 μmol/l.

The effects of the parent aglycones and equol on lag phase duration are compared with the effects of their sulphated conjugates in Fig. 4. Influences of sulphation at the 4′ hydroxyl group, the 7 hydroxyl group and at both groups are shown. Again, compounds are ordered according to the size of the effect. It is clear that in every case the conjugation reduced antioxidant activity. Furthermore, the 4′ monosulphates were less effective than the 7 monosulphates, and the disulphates were less effective than either. In fact the disulphates had no real effect on the lag phase even at the highest concentration (5 μmol/l).

The effects of 8-OH-daidzein and equol disulphate on the electrophoretic mobility of LDL are shown in Fig. 5. The gel illustrates the results of incubating with copper and/or the test compounds for 3 h, using the higher concentrations of LDL, copper and test compound described above. Identical trends were obtained at 4, 5 and 6 h, and at the lower concentrations (data not shown). As expected, the mobility of LDL incubated with copper (lane 3) was greater than non-oxidised LDL (lane 1), consistent with the increased negative charge normally observed during LDL oxidation. Incubating the LDL with 8-OH-daidzein but without any oxidative stimulus (lane 2) gave a result that was identical to the non-oxidised LDL; hence the 8-OH-daidzein does not directly alter mobility, for example by binding to the LDL. Incubating the LDL with 8-OH-daidzein in addition to the copper (lane 4) again gave a result identical to the non-oxidised LDL (lane 1), consistent with 8-OH-daidzein completely preventing oxidative modification of the apolipoprotein B100. When this experiment was repeated with equol disulphate, however, the LDL (lane 5) had identical mobility to LDL incubated with copper but no test

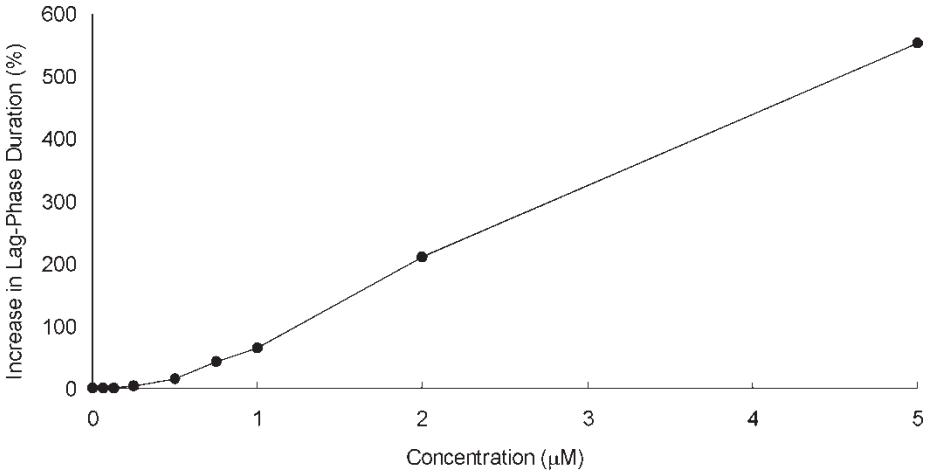

FIGURE 3 Effect of 8-OH-daidzein on the duration of the lag phase during oxidation of LDL by copper, with data extending to lower concentrations than shown in Fig. 2. Points show means ± SEM's of triplicate experiments. (SEM's are too small to see in many cases.)

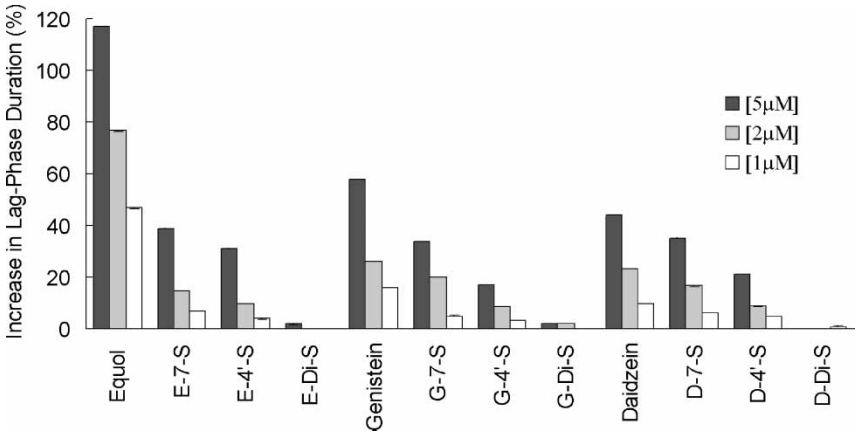

FIGURE 4 Effects of genistein, daidzein, equol and their sulphated conjugates, at concentrations of 1, 2 and 5 μmol/l, on the duration of the lag phase during oxidation of LDL by copper. Data were obtained for each parent compound, its 4′- and 7-sulphates and its di-sulphate. Bars show means ± SEM's of triplicate experiments. (SEM's are too small to see in many cases.)

compound (lane 3), showing that under the same conditions equol disulphate had no influence on oxidation.

DISCUSSION

Soy isoflavones are thought to have a cardioprotective effect that is partly mediated by an inhibitory influence on the oxidation of LDL. The aim of the present work was to investigate whether the antioxidant properties attributed to soy isoflavones apply to the forms that actually circulate in appreciable quantities, as well as to the widely available but less relevant compounds examined in many previous studies. Equol and *O*-DMA were studied as examples of the products of further gut metabolism, the mono- and di-sulphates of genistein, daidzein and equol as examples of liver and gut conjugates, and 8-OH-daidzein as an example of the hydroxylated forms produced in the liver. Genistein and daidzein, the parent aglycones, were included to allow comparison with their metabolites, and quercetin was used as a positive control. This is the first study examining effects of the isoflavone conjugates, yet these compounds are of critical importance. Only 3% of daidzein, for example, is excreted in urine in the free form, the large majority being conjugated (81% monoglucuronide, 1% diglucuronide, 3% monosulphate, 2% disulphate and 10% sulphoglucuronide).[22]

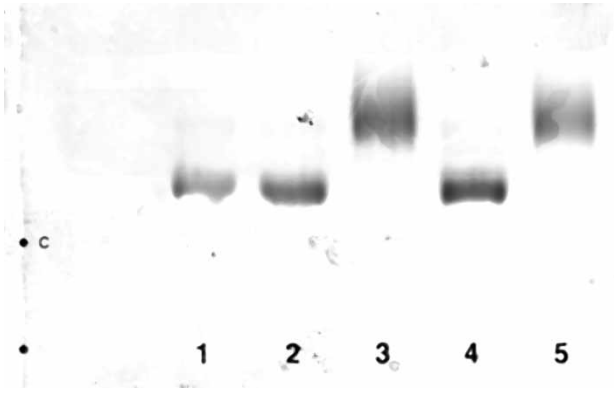

FIGURE 5 Agarose gel electrophoresis of LDL incubated in the absence or presence of copper and in the absence or presence of the test compounds 8-OH-daidzein or equol disulphate. The incubation lasted 3 h, and concentrations were: LDL, 500 μg protein/ml; Cu^{2+} ions and test compounds, 50 μmol/l. The gel was stained for lipid with Paragon® lipoprotein stain. Lane 1: LDL incubated in the absence of copper and test compound. Lane 2: LDL incubated with 8-OH-daidzein in the absence of copper. Lane 3: LDL incubated with copper alone. Lane 4: LDL incubated with copper and 8-OH-daidzein. Lane 5: LDL incubated with copper and equol disulphate. 8-OH-daidzein abolished the normal oxidation-induced increase in net negative charge, but equol disulphate had no effect. Identical trends were seen at incubations lasting 4, 5 or 6 h, and when the concentrations of all compounds in the incubation mixtures were reduced 10-fold. "C" indicates the origin.

The influence of the compounds on the oxidation of LDL was examined since it represents one of the most important anti-atherogenic properties ascribed to them; there is considerable evidence that such oxidation is a key process in the development of the disease.[23,24] When LDL is exposed to copper (II) ions, the appearance of oxidised species shows a predictable pattern. Oxidation of polyunsaturated fatty acids and the consequent formation of conjugated dienes is an early step that can be conveniently followed in a spectrophotometer.[21] The appearance of a significant rise in the concentration of conjugated dienes is preceded by a lag phase, during which endogenous and exogenous antioxidants are themselves oxidised whilst preventing the oxidation of the LDL lipids. The duration of

this lag phase is an indication of the antioxidant capacity of the LDL and its surrounding milieu[25] and an increase in the lag phase *in vivo* might slow the initiation and development of lesions. (The mechanisms by which LDL is oxidised *in vivo* are the subject of controversy, but copper-mediated oxidation is a plausible contributory pathway because catalytically-active copper capable of oxidising LDL is present in lesions.[26])

In this study, as in previous ones, genistein and daidzein significantly increased the duration of the lag phase, although the effect was only around 5% that of quercetin. Previous studies of antioxidant activity[2,27,28] have generally found genistein to be more effective than daidzein, a trend also seen here, suggesting that the C5 hydroxyl group on genistein plays a significant antioxidant role. The main finding of the present study was that metabolism of these parent aglycones can increase or decrease their antioxidant effect. Importantly, effects on lag phase were observed, and the trends caused by metabolism were apparent, at 1 μmol/l, a physiologically achievable plasma isoflavone concentration in people on Eastern diets. The gut metabolite equol and the liver metabolite 8-OH-daidzein were, respectively, 2.65-fold and 12.5-fold more effective than their parent compound, daidzein. Indeed the effect of 8-OH-daidzein was >50% that of quercetin. Conversely, *O*-DMA, another gut metabolite, had a smaller effect than daidzein. Considerable attention has focused on equol because it has higher antioxidant capacity than its parent compound.[28,29] The novel result that 8-OH-daidzein is approximately 5-fold more potent at inhibiting LDL oxidation suggests that this metabolite could have even greater significance.

It is instructive to compare these results with data from our recent study[30] in which more fundamental measures of the antioxidant activity of isoflavone metabolites were obtained. In this earlier work, 8-OH-daidzein was better at scavenging hydroxyl radicals than were genistein, daidzein, equol and *O*-DMA, and when superoxide anion radical scavenging was examined, only 8-OH-daidzein had any effect. Scavenging of the 2,2′-azino-*bis*-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) radical was greatest for *O*-DMA and equol, followed by 8-OH-daidzein, genistein and daidzein. When reduction of Fe^{3+} to Fe^{2+} was studied, 8-OH-daidzein was the most potent, followed by equol and *O*-DMA; genistein was considerably less effective than daidzein. Although the order of potency was dependent to some extent on the assay used (as in earlier investigations[27,28]), some broad trends can be discerned. Genistein and daidzein were relatively ineffective, and 8-OH-daidzein was generally highly effective, both at scavenging free radicals and at preventing oxidation induced by metal ions. The ranking of equol and *O*-DMA were more variable; *O*-DMA in particular was sometimes highly potent, consistent with the earlier result of Hodgson *et al.*,[29] and sometimes less effective, as here.

The most important finding of the present study was that conjugation with sulphate groups decreased the antioxidant effect of genistein, daidzein and equol. This result is significant because almost all circulating isoflavones are conjugated with sulphate or glucuronate. (We speculate that the glucuronides, had they been available, would have shown the same behaviour as the sulphates since the same hydroxyl groups are affected.) A notable trend was that antioxidant capacity was consistently in the order: parent compound > 7 monosulphate >4′ monosulphate > disulphate regardless of whether the sulphates were conjugated to genistein, daidzein or equol. In fact, the antioxidant power of the disulphates was negligible. It seems likely that the loss of antioxidant activity is related to the removal by the sulphate groups of the phenolic hydroxyl groups. A corollary of this is that the hydroxyl group at the 4′ position must be more effective than that at the 7 position. This conclusion is consistent with previous data obtained by comparing naturally-occurring isoflavone glycosides with their aglycones: genistin and daidzin had approximately the same antioxidant activity as genistein and daidzein[28] despite the blockage of the C7 hydroxyl group by glucose, whereas biochanin A and formononetin, in which the 4′ hydroxyls of the aglycones are blocked by a methoxy group, were substantially less effective.[27,28] In the present study, however, this conclusion is more robust since it is based on comparison between conjugates having the same substituent group, and the observation is extended for the first time to physiologically-relevant compounds.

Although the main focus of this study was the effect of isoflavones and their metabolites on the duration of the lag phase, the effects of selected compounds on the charge of the LDL particle were also examined. During oxidation, the apolipoprotein B100 moiety of LDL becomes more negatively charged. Changes to the apolipoprotein are important because they affect receptor binding of the LDL particle. In particular, recognition of oxidised LDL by scavenger receptors, a property not shared with native LDL, leads to unregulated cellular uptake and is one mechanism by which the foam cells characteristic of early atherosclerotic lesions may develop.[23]

Effects of the isoflavones and their metabolites on charge were expected as a consequence of their influence on lipid peroxidation. However, it is conceivable that the compounds could also have effects on charge not related to their effects on lipid peroxidation, perhaps mediated by direct interaction

with the apolipoprotein. In that case, the relative influence of the various compounds on charge could well differ from their relative effect on the lag phase. For example, it is plausible that the disulphates, despite being ineffective in slowing lipid peroxidation, could still retard the change in charge.

Of the metabolites, 8-OH-daidzein and equol disulphate were chosen for this further study because the former produced the greatest increase in lag phase whilst the latter, despite being a metabolite of the same parent compound, had no effect. Their influence during periods of copper-mediated oxidation ranging from 3 to 6 h was measured. Electrophoresis of the LDL indicated that at all durations (and at the two concentrations examined), 8-OH-daidzein effectively abolished the change in charge whilst equol disulphate was without influence. Thus their effects on charge were entirely consistent with their effects on lipid peroxidation; the data do not provide evidence for any direct actions of these compounds on subsequent processes taking place during LDL oxidation.

In conclusion, metabolism in the gut and liver significantly altered the antioxidant activity of soy isoflavones and is therefore likely to affect their anti-atherogenic and other health-promoting effects. Equol was substantially more effective than its parent compound daidzein, as previously established, and 8-OH-daidzein was more potent still. If, as this result suggests, hydroxylation in the liver increases antioxidant capacity then the di-hydroxyl derivatives may be even better antioxidants. However, it was also found that the conjugates formed in the liver and the mucosa of the small intestine have reduced effectiveness. The significance of this observation derives from the fact that most circulating isoflavones are conjugated. Hence the reduced effectiveness of the conjugates *in vitro* is consistent with the fact that only a small increase in the duration of the lag phase (4 min, or approximately 8%) was produced by dietary isoflavones in a human intervention study.[31] Di-sulphation resulted in complete abrogation of antioxidant activity. This was true even in the case of equol, despite the notable effectiveness of the unconjugated form *in vitro*. An unresolved question is whether disulphates of the hydroxylated metabolites produced in the liver would be similarly ineffective. In these compounds, the hydroxyl groups are added in the ortho position of the phenolic hydroxyl groups and would not be directly masked by sulphate or glucuronate groups during conjugation. The antioxidant capacity of such conjugated compounds, should they become available, would be an important area for further research.

Acknowledgements

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