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Genistein Prevents the Glucose Autoxidation Mediated Atherogenic Modification of Low Density Lipoprotein

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Hyperglycemia has been assumed to be responsible for oxidative stress in diabetes. In this respect, glucose autoxidation and advanced glycation end products (AGE) may play a causal role in the etiology of diabetic complications as e.g. atherosclerosis. There is now growing evidence that the oxidative modification of LDL plays a potential role in atherogenesis. Glucose derived oxidants have been shown to peroxidise LDL. In the present study, genistein, a compound derived from soy with a flavonoid chemical structure (4', 5, 7-trihydroxyisoflavone) has been evaluated for its ability to act as an antioxidant against the atherogenic modification of LDL by glucose autoxidation radical products. Daidzein, (4', 7-dihydroxyisoflavone) an other phytoestrogen of soy, was tested in parallel. Genistein – in contrast to daidzein – effectively prevented the glucose mediated LDL oxidation as measured by thiobarbituric acid-reactive substance formation (TBARS), alteration in electrophoretic mobility, lipid hydroperoxides and fluorescence quenching of tryptophan residues of the lipoprotein. In addition the potential of glucose-oxidized LDL to increase tissue factor (TF) synthesis in human endothelial cells (HUVEC) was completely inhibited when genistein was present during LDL oxidative modification by glucose. Both phytoestrogens did not influence the nonenzymatic protein glycation reaction as measured by the in vitro formation of glycated LDL. As the protective effect of genistein on LDL atherogenic modification was found at glucose/genistein molar ratios which may occur in vivo, our findings support the suggested beneficial action of a soy diet in preventing chronic vascular diseases and early atherogenic events.

Keywords: glucose autoxidation, LDL oxidation, genistein, daidzein, soy phytoestrogen

INTRODUCTION

It has been assumed that oxidative stress contributes to the development of the diabetic complications [1]. In this respect, lower levels of antioxidants like ascorbic acid and vitamin E have been reported to occur in diabetes [2,3]. Hyperglycemia may cause oxidative stress, but there is still a debate regarding the pathogenic link between hyperglycemia and complications [1]. Elevated levels of glycoxidation and lipoxidation products have been found in plasma and tissue proteins in diabetes [4–7]. Insulin treatment of type 1 diabetes patients reduced plasma

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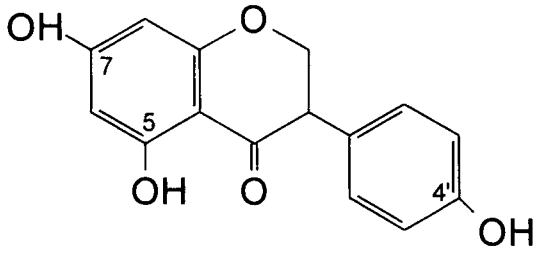
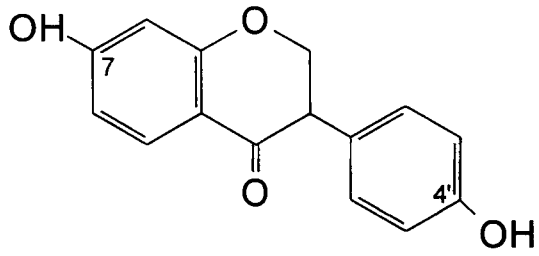
hydroperoxides and, thus, reactive oxygen species as reported by [8].

The onset and progression of atherosclerosis is accelerated in diabetes [9]. LDL oxidative (atherogenic) modification has been found to play a pivotal role in early stage atherogenesis [10–12]. Glucose treatment of LDL results in glycosylation and in peroxidation of the lipoprotein [13–15]. Recently we have shown that the phytoestrogen genistein, which has been found to be a potent inhibitor of endothelial cell proliferation and angiogenesis [16,17], is an efficacious antioxidant of the endothelial cell mediated oxidation of LDL [18]. The aim of this study was to assess the potential of the soy diet isoflavones genistein and daidzein (see Scheme 1) to act as inhibitors of the glucose mediated atherogenic LDL modification.



Genistein



Daidzein

SCHEME 1 Chemical structures of genistein and daidzein

MATERIALS AND METHODS

Materials

Genistein (4',5,7-trihydroxyisoflavone), daidzein (4',7-dihydroxyisoflavone), bovine serum albumin and lipopolysaccharide (LPS, E. coli 055:B5) were from Sigma Chemical Co., Germany. D-glucose monohydrate was from Merck, Germany. All other chemicals used were of analytical grade.

LDL Isolation

LDL was isolated from EDTA-plasma of healthy male volunteers by ultracentrifugation as reported previously [19]. The final preparation was dialyzed against 150 mmol/L NaCl containing 0.1 mmol/L EDTA and filter-sterilized.

LDL Oxidation by Glucose

Prior to LDL oxidation the lipoprotein was equilibrated in 0.1 mol/L phosphate buffer pH 7.4 using Sephadex G-25 chromatography (PD-10 columns, Pharmacia, Sweden). LDL (2mg/ml) was incubated in the presence of 200 mmol/L D-glucose [7,15] up to 9 days at 37° under sterile conditions.

Cell Culture

Endothelial cells (HUVEC) were isolated from human umbilical veins and maintained in culture as reported previously [20].

Analysis of LDL Oxidation

Thiobarbituric acid assay

LDL oxidation products were assayed as TBARS as described [19].

Lipid hydroperoxide assay

Total lipid hydroperoxides were assayed based on the method of El Saadani et al [21] as modi-

fied by Wallin and Camejo [22]. The concentration of hydroperoxides was calculated from the molar absorption coefficient of 2.46×10^4 mol/L $\times$ cm^{-1} at 365 nm. Prior to the estimation of lipid hydroperoxides the LDL samples were applied to small Sephadex G-25 columns (Nick columns, Pharmacia, Sweden) to remove low molecular weight compounds [23], which can interfere with lipid hydroperoxide determination.

Lipid electrophoresis

Aliquots (10 µl) of treated or untreated LDL were applied to agarose gels (1% in veronal buffer) and run for 90 minutes and lipoproteins were detected according to the supplier of the analytical system (Lipidophor All In, Immuno-Baxter AG, Austria). Measurement of relative electrophoretic mobility (REM) was taken as an indicator of LDL oxidation [24], setting the electrophoretic mobility of native (untreated) LDL arbitrarily as 1.

Tissue Factor – Clotting Assay

Confluent HUVEC cultures grown in 6-well plates were washed with RPMI 1640 and treated for 4 to 5 hours with the respective LDL preparations (100 µg/ml medium) to stimulate tissue factor production [25]. At the end of incubation cells were washed with PBS. HUVEC were scrape-harvested into 1 ml of PBS and sonicated by a cell-disrupter for 1 minute at 4°C, the lysate was assayed in an one-stage clotting assay: 100 µl citrated normal donor platelet poor plasma was incubated for 1 minute with 100 µl cell-lysate at 37°C in prewarmed plastic tubes of a KC-10 coagulometer (Amelung, Germany); 100 µl $CaCl_2$ (30 mmol/L) were than added and coagulation time was measured. Control experiments with factor VII, IX and X deficient plasma (Behring, Germany) characterized the procoagulant activity as TF [20].

Fluorescence Measurement

Glucose treated or untreated LDL or bovine serum albumin incubation mixtures were applied to Sephadex G-25 columns equilibrated in 0.025 mol/L phosphate buffer pH 7.4 to get rid of low molecular weight compounds and subsequently the fluorescence of the protein samples was estimated in a Hitachi 650–10S Fluorescence Spectrophotometer (Japan).

LDL Glycation

LDL (1 mg/mL) was incubated in 0.1 mol/L phosphate buffer, pH 7.4 in presence of 20 mmol/L D-glucose and 10 µCi/mL U-^{14}C glucose (ICN Biomedicals, USA, specific activity 316 mCi/mmol) at 37°C for 6 days. Lipoproteins were precipitated by the addition of trichloro acetic acid (TCA) to a final concentration of 5 %. The pellet was washed in 5 % TCA and finally dissolved in formic acid before scintillation counting [14].

Hemoglobin Glycation

PBS-washed erythrocytes were lysed by the addition of distilled water and incubated with gentle mixing at 4°C for 60 minutes. After centrifugation for 10 min. at 14.000 $\times$ g the supernatant was taken as the source of hemoglobin. For in vitro hemoglobin glycation the preparations (100 mg/ml in 0.1 mol/L phosphate buffer pH 7.4) were incubated in the absence or presence of glucose (200 mmol/L) and the respective compounds for 10 days at 37°C.

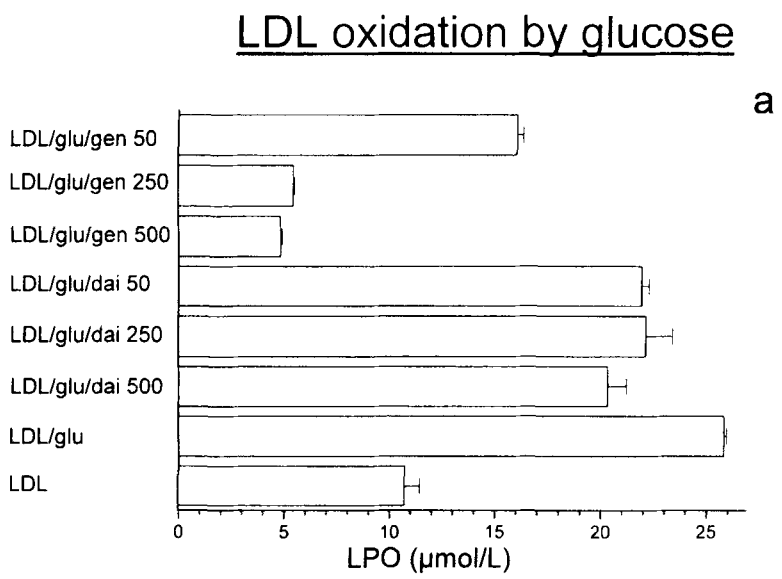
Hemoglobin and HbA_{1c} Measurement

Hemoglobin was estimated on a Hitachi 911 analyzer (Roche, Switzerland) and hemoglobin glycation was measured using a commercial HbA_{1c} test-kit (Roche, Switzerland) based on immuno-turbidimetry.

RESULTS

To examine the potential of the soy diet phytoestrogens genistein and daidzein to prevent the atherogenic (oxidative) modification of LDL by the various reactive oxygen species generated by glucose autoxidation, LDL (2mg/ml) was incubated in presence of glucose (200 mmol/L) with or without the respective phytoestrogen. As can be seen in figure 1a when LDL was incubated with glucose for 4 days at 37°C there was a more than doubling of lipid hydroperoxides monitored compared to controls without glucose. The presence of daidzein during glucose oxidation in concentrations of 50, 250 and 500 μmol/L had only a marginal effect on LDL oxidation. However, genistein exerted a strong inhibitory action on glucose autoxidation mediated LDL atherogenic modification (see figure 1a). At the lowest genistein concentration tested (50 μmol/L) the phytoestrogen suppressed lipid hydroperoxide production to 15 μmol/L (61%) compared to lipid hydroperoxides in presence of high glucose (26 μmol/L, 100%). This different antioxidant potential of the isoflavones was also reflected when TBARS were estimated in the respective LDL samples. As depicted in figure 1b, genistein was the most active compound to suppress TBARS formation in glucose treated LDL. As a further measure of LDL oxidative modification quenching of tryptophan fluorescence [27–30] was monitored in LDL samples subjected to oxidation by glucose. As can be seen in figure 2a treatment of LDL (2mg/ml) by glucose (200 mmol/L) resulted in about 30% quenching of the tryptophan fluorescence. The presence of daidzein (50, 250 and 500 μmol/L) in the LDL/glucose incubation mixtures had no effect on fluorescence quenching (figure 2a). In contrast, genistein prevented quenching of tryptophan fluorescence at 500 and 250 μmol/L and even in the lowest concentration (50 μmol/L) genistein exerted some protective effect (figure 2b). The influence of daidzein and genistein on glucose autoxidation reactive products on protein modification was additionally studied using BSA as a model protein [30,31]. Incubation of BSA (2mg/ml) in presence of glucose (200 mmol/L) for 14 days at 37°C resulted in fluorophor development. As presented in figure 3, relative fluorescence increased from 1.2 to 5.8 for untreated and glucose treated albumin, respectively (excitation 350 nm, emission 420 nm). Daidzein (500 μmol/L) in the albumin/glucose incubations had a modest influence on fluorescence development (relative fluorescence 4.9 versus 5.8). However, 500 μmol/L genistein very effectively inhibited protein modification by glucose (relative fluorescence 1.7 versus 5.8). The increase of fluorescent material in albumin was suppressed by genistein (figure 3) almost reaching fluorescence values of non-glucose treated albumin (relative fluorescence 1.8 vs. 1.2).

Oxidized LDL has been shown to induce tissue factor production in endothelial cells [25,32]. Thus we have tested the ability of both phytoestrogens to prevent the modification of LDL by glucose autoxidation products to a lipoprotein particle able to stimulate tissue factor synthesis in endothelial cells. When LDL (2mg/ml) was incubated in presence of glucose (200 mmol/L) for 9 days at 37°C and subsequently HUVEC cultures were treated with this LDL preparation (100 μg/ml medium) for 4 hours, tissue factor was induced as assessed by clotting assay (see Figure 4). Tissue factor induction could be prevented if genistein (500 μmol/l), but not when daidzein was present during glucose-mediated LDL oxidation. As seen in Figure 5a-c the very different ability of daidzein and genistein to overcome the effect of glucose treatment on LDL in respect to tissue factor synthesis in HUVEC cultures was also reflected in their antioxidant potential under these LDL/glucose incubation conditions (9 days at 37°C).



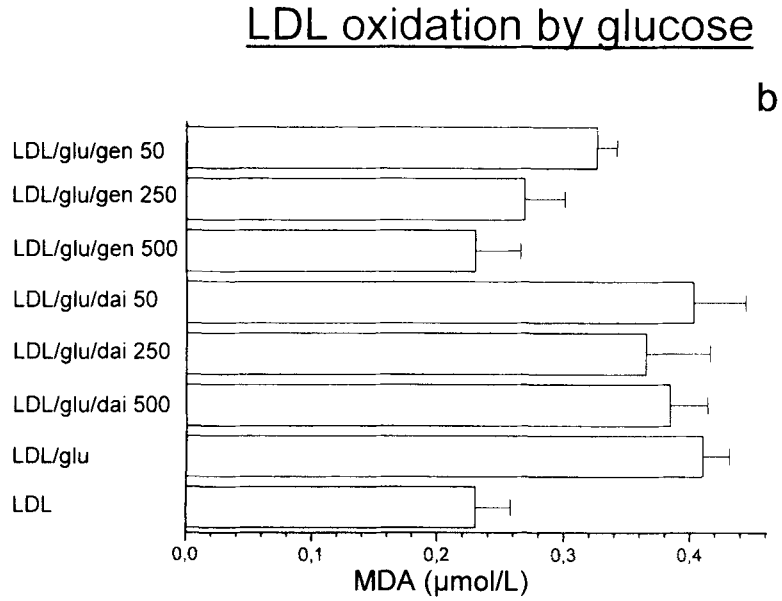


FIGURE 1 Influence of genistein and daidzein on glucose mediated LDL oxidation. LDL (2 mg/ml) was incubated in phosphate buffer in presence of glucose (200 mmol/L) with or without the respective concentration of genistein or daidzein for 4 days at 37°C under sterile conditions. Subsequently the lipid hydroperoxides (a) and TBARS (b) were estimated as given under methods. Mean values and standard deviation (error bars) are given

Glycated LDL has been shown to be more prone to oxidation than unmodified LDL. Thus, one may assume that genistein may inhibit LDL glycation resulting in less LDL oxidation. Incu-

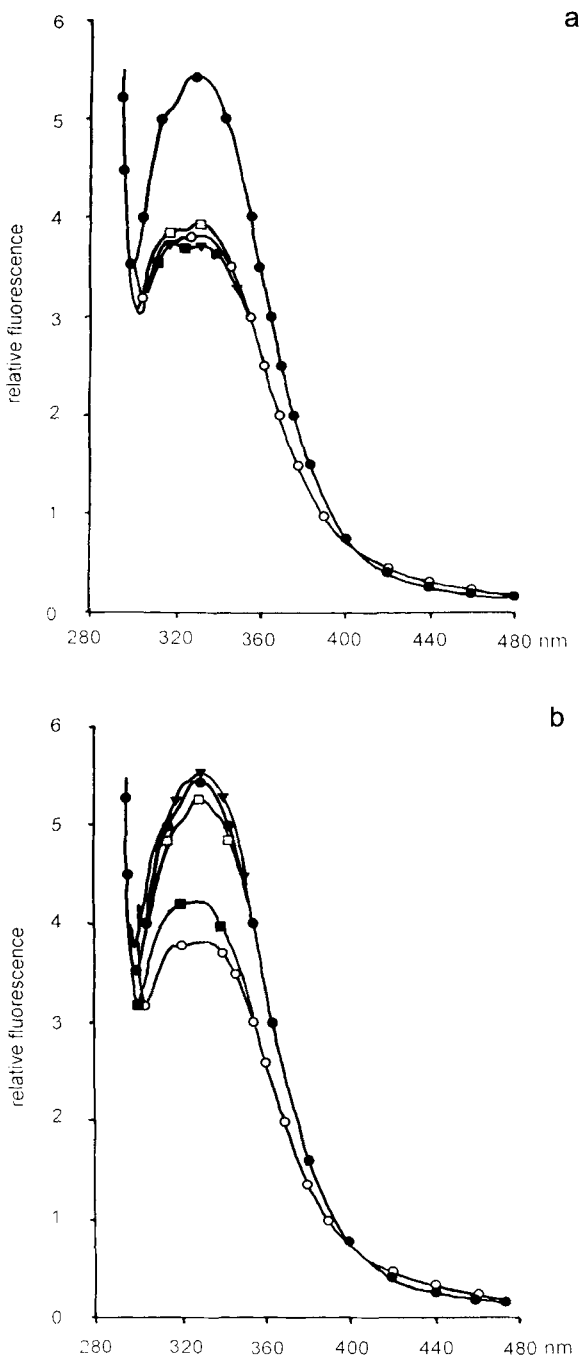


FIGURE 2 Influence of genistein and daidzein on glucose-mediated tryptophan fluorescence quenching in LDL. LDL (2 mg/ml) was incubated in phosphate buffer in presence of glucose (200 mmol/L) with or without the respective concentration of genistein or daidzein for 4 days at 37°C under sterile conditions. Subsequently tryptophan fluorescence (excitation 280 nm) was monitored as given under methods. a: daidzein. b: genistein. ■: LDL/glucose + 50 µmol/L compound, ▼: LDL/glucose + 200 µmol/L compound, □: LDL/glucose + 500 µmol/L compound, ●: LDL alone, ○: LDL/glucose

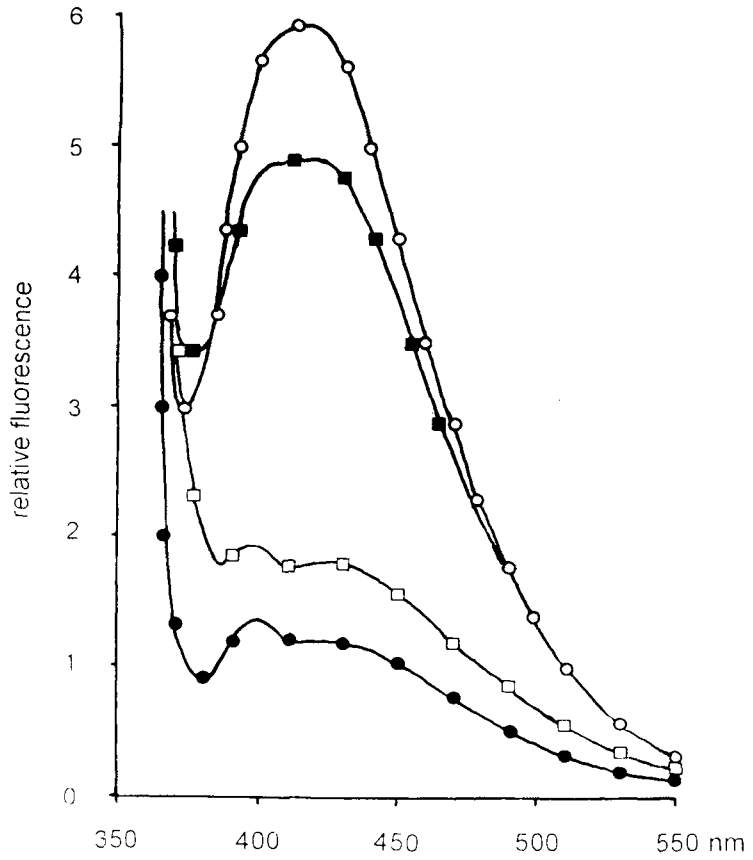


FIGURE 3 Influence of genistein and daidzein on fluorophor development in albumin. Bovine serum albumin (2 mg/ml) was incubated in phosphate buffer in presence of glucose (200 mmol/L) with or without the respective concentration of genistein or daidzein for 14 days at 37°C under sterile conditions. Subsequently fluorescence development (excitation 350 nm) was monitored as given under methods. ●: albumin, ○: albumin/glucose, ■: albumin/glucose + 500 µmol/L daidzein, □: albumin/glucose + 500 µmol/L genistein

bation of LDL with ^{14}C glucose resulted in the binding of 13.7 ± 0.87 nmol glucose/mg LDL. Neither genistein nor daidzein (molar ratio glucose/compound = 400) inhibited LDL glycation (14.1 ± 1.51 and 14.0 ± 1.32 nmol glucose/mg LDL, respectively). The well known hemoglobin glycation reaction was also not interfered by the phytoestrogens (data not shown). Transition metal ions can catalyze glucose oxidation [30,31] and chelation of such ions may therefore result in reduced LDL oxidation by glucose. However, spectral analysis revealed that no complex formation occurred between Fe^{++} or $Cu^{++}$and the respective phytoestrogen (results not shown).

DISCUSSION

Autoxidation of glucose generates a battery of free radical species such as superoxide-, hydroxyl-, hydroxyalkyl-, and peroxyl radicals as well as hydrogen peroxide [30,31]. Thus glucose, beside its ability to cause glycation of sev-

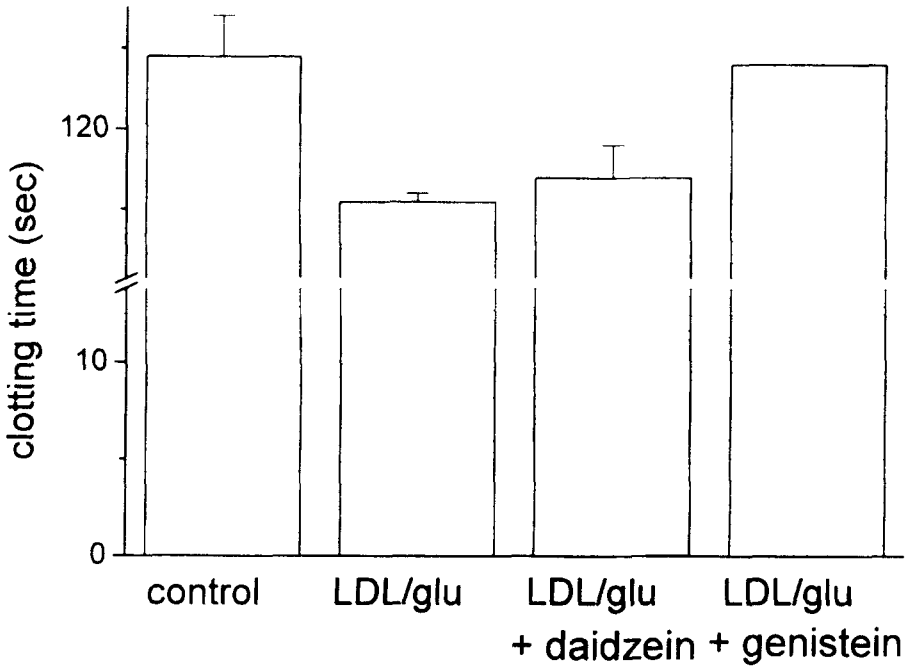


FIGURE 4 Tissue factor coagulant activity in human endothelial cells. HUVEC cultures were exposed for 4 hours to the respective LDL preparations (100 μg/ml) and subsequently the tissue factor coagulant activity was estimated in aliquots of HUVEC protein extracts as given under methods. glu = glucose. Mean values and standard deviation (error bars) are given

eral proteins via aldehyde-amino group reactions (Schiff base formation), exerts oxidative attacks to proteins as well as lipids [7,30,31]. These attacks result in protein fragmentation and peroxidation of lipids generating new species of reactive, cytotoxic compounds [31,33].

LDL exposed to glucose is known to undergo peroxidation of the lipid moiety and glycation of the apoprotein. [13–15]. These modifications results in a lipoprotein particle known to initiate and support atherogenic events [34–36].

Glycated LDL, which has been detected in plasma of diabetic patients, can also impair the vasodilatory response and is even more prone to oxidation than native LDL [13–15, 37,38]. Taken all these observations into account antioxidative compounds [39–42] may have beneficial effects regarding diabetic complications. The natural antioxidants like flavones, isoflavones, catechins etc. have attracted most attention [43–50]. In this respect we have recently reported that the soy diet isoflavone genistein acts as a potent antioxidant against endothelial cell mediated LDL oxidation and in addition protected vascular cells from the cytotoxic attack of atherogenic LDL [18].

In the present study we now show that genistein but not daidzein effectively counteracted the oxidative modification of LDL by glucose. The inability of daidzein to act as an antioxidant in this particular LDL oxidation system may be due to its chemical structure as a diphenol whereas genistein is a triphenolic compound with a 5,7 dihydroxy structure in the A ring (see Scheme 1). Such structural arrangements of the phenolic groups have been found to confer the antioxidant activity of the phytoestrogenic isoflavones [50]. In other LDL oxidizing systems daidzein

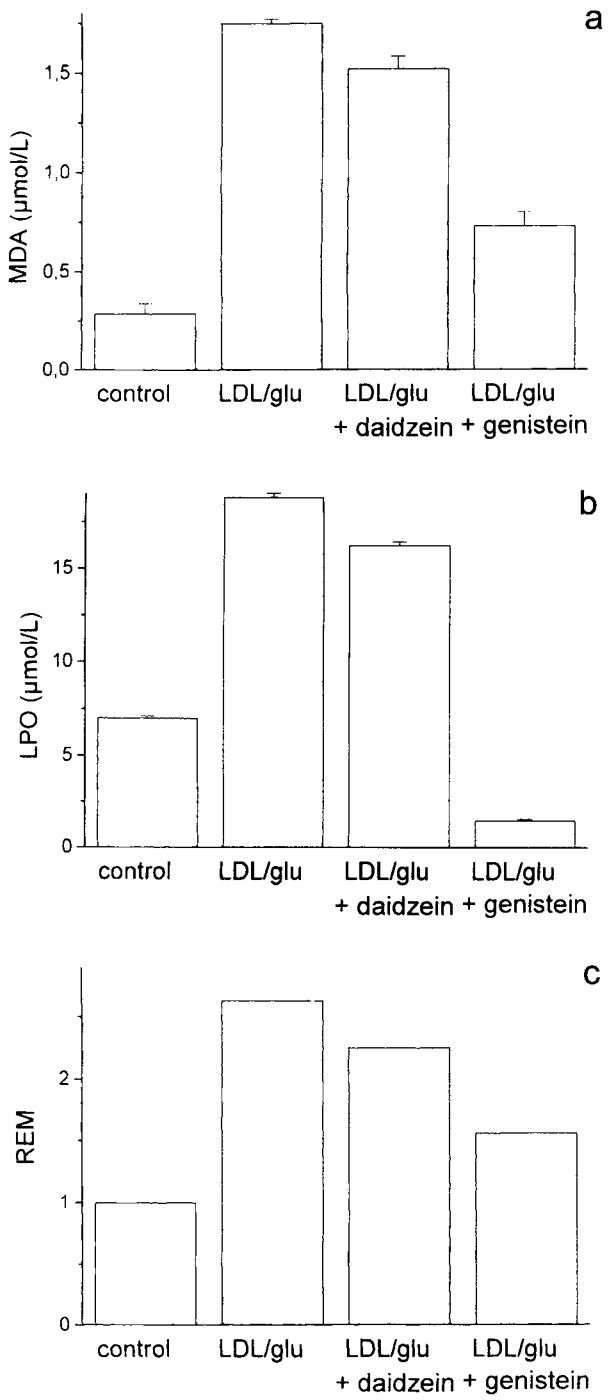


FIGURE 5 Influence of genistein and daidzein on LDL glucose preparations used for tissue factor activity induction in endothelial cells. LDL (2 mg/ml) was incubated in phosphate buffer in presence of glucose (200 mmol/L) with or without the respective concentration of genistein or daidzein (500 µmol/L each) for 9 days at 37°C under sterile conditions. Subsequently malondialdehyde (5a), lipid hydroperoxides (5b) and REM (5c) were estimated as given under methods. glu = glucose

was also less effective in comparison to genistein [18,50]. In humans consuming soy products, genistein (and daidzein) can be found in plasma [51,52]. *In vivo* a molar ratio of glucose/genistein of about 1000 (in normoglycemic individuals) can be reached. The molar ratios of 800 to 4000 used in our *in vitro* system inhibited LDL oxidation. The results indicate that genistein may act solely as a free radical scavenger and not by binding catalytically active metal ions or inhibiting the formation of oxidation prone glycated LDL as no transition metal ion chelation activity (this study and [50]) or inhibition of the non enzymatic glycosylation of LDL was observed by the compound.

In summary our results show that from both main isoflavonoids found in soy, genistein but not daidzein was an efficacious antioxidant in respect to LDL modification by glucose derived radical products. The compound inhibited the formation of atherogenic LDL particles at glucose/genistein molar ratios which may be found in vivo. This findings will support the suggested and documented beneficial action of a soy diet in preventing chronic vascular diseases and early atherogenic events [53–55]. However it should be kept in mind that hyperglycemia is the first link in the pathogenesis of diabetic complications requiring primary attention [1] and antioxidant therapy [56] may be an accompanying one. On the other hand the benefit of antioxidant supplementation regarding prevention of atherosclerosis is not as clear as one may desire as paradoxical actions of antioxidants can not be ruled out [57]

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