Clinical Research

Isoflavone genistein protected high glucose-induced human aortic endothelial cell apoptosis through estrogen receptor-mediated pathway

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Objective The aim of this study was to determine if isoflavone genistein had protective effects against high glucose-induced cell apoptosis in human aortic endothelial cells, and investigate the possible mechanism for this protection. Methods Human aortic endothelial cells subjected to normal (5mM) or high glucose (25mM) were treated with genistein at 0, 50, 100nM. Parallel experiments were performed with 100nM 17b-estradiol, and also in the presence and absence of the pure anti-estrogen ICI-182,780 (100nM). The effects on cell apoptotic DNA fragmentation were determined using cell death ELISA, and the effects on cellular proliferation were determined using tritiated thymidine incorporation assay. Estrogen receptor expression was detected by Taqman quantitative PCR. Results 100nM Genistein significantly reduced high glucose-induced DNA fragmentation, and reversed cell DNA synthesis inhibition \( (P < 0.001) \) after 24 hours' incubation. The effect of genistein was completely blocked by ICI-182,780 administration. Estrogen receptor beta, but not alpha was found to be expressed in these cells. Conclusion Isoflavone genistein gave protection against high glucose-induced cell damage through estrogen receptor beta, reducing apoptotic DNA damage and protecting from the inhibition of cell proliferation. (J Geriatr Cardiol 2008; 5: )

Key Words phytoestrogen; endothelial cell; high glucose; estrogen receptor; apoptosis

Introduction

Phytoestrogens are a group of biologically active plant substances with chemical structures similar to the endogenous estrogen, allowing them to bind to estrogen receptors (ER), though with lower affinity. Epidemiologically, there is a lower incidence of cardiovascular disease in Asian countries that have a high soy phytoestrogen consumption in their diet. The anti-oxidative properties of phytoestrogens may be responsible in part for their potential protective effects. Genistein at physiological concentrations reduced free radicals damage to MC3T3-E1 cells more effectively than either vitamin E or C, glucose-triggered oxidation of LDL can be effectively prevented by genistein; genistein and equol were shown to protect against \( \mathrm{H}_2\mathrm{O}_2 \)-induced DNA damage in human lymphocytes, more than anti-oxidant vitamins and estradiol. Genistein also plays a role in the scavenging of reactive oxygen species and lipid peroxidation. Daidzein has been shown to increase catalase mRNA expression and activate the catalase promoter region, and phytoestrogens can increase total glutathione levels, thus decreasing intracellular oxidant levels and preventing oxidative DNA damage.

The studies performed here were to determine the protection from high glucose induced oxidative stress by the isoflavone genistein on human aortic endothelial cells (HAnECs), and to determine if these effects were through the estrogen receptor or via an alternative pathway.

Materials and Methods

Reagents

All reagents and chemicals were obtained from Sigma, Poole, UK unless otherwise stated. All reagents for tissue culture were obtained from Invitrogen, UK. The cell death ELISA was obtained from Roche Diagnostics Ltd.

Cell culture

HAnECs were purchased from PromCel Ltd, and cultured in endothelial cell growth media (phenol red free) that was supplemented with 2% fetal calf serum, 5.0 ng/ml epidermal growth factor, 0.5 ng/ml vascular endothelial growth factor, 10 ng/ml basic fibroblast factor, 20 ng/ml R3 IGF-1, 22.5 i g/ml heparin, 50 ng/ml amphotericin B and 50 i g/ml gentamicin. This medium kit initially contained supplements of ascorbic acid and hydrocortisone, which were omitted for this study, and the concentration of D-glucose is 5 mM. Cells were seeded into culture plates at the density of 2. 5×10^5/ml and incubated at 37°C in an atmosphere of 5% CO2. Control cells received normal 5mM glucose medium;
high D-glucose treated cells had this medium supplemented with D-glucose to achieve 11.3 or 25mM glucose in total. Cells with or without the pure anti-estrogen ICI182,780 (100nM) received genistein or 17-estradiol (1nM) for 24 hours. All experiments were performed in triplicates.

**Cell Death Detection ELISA**

To detect the DNA fragmentation of apoptotic cells, the Cell Death Detection ELISA assay was used according to the manufacturer’s protocol. Briefly, cells were lysed with 200ìl lysis buffer and centrifuged at 4,000rpm for 10min, and 20ìl supernatant of each sample was transferred into a streptavidin-coated microtitre plate (MTP). 80ìl of the immunoreagent mixture was added, washed and 100ìl ABTs substrate solution added. A MTP reader (Anthos 2010, Anthos Labtech Instruments) was used to measure the absorbance at the wavelength of 405nm with 492nm as a reference.

**Cytotoxicity Detection Assay LDH**

Necrosis was determined through the activity of Lactate Dehydrogenase (LDH) released from the cytosol; the Cytotoxicity Detection Kit (Roche Diagnostics Ltd, UK) was used according to the manufacturer’s protocol. Briefly, 100ìl culture supernatant of each sample was transferred into a 96-well plate, followed by adding 100ìl reaction mixture to each well. The samples were incubated for 20 minutes in the dark at room temperature and the absorbance was measured by the MTP reader at the wavelength of 492 nm with 602 nm as a reference.

**Tritiated Thymidine incorporation assay**

Each sample received 0.25uCi of [3H]-TdR (Amersham International, Amersham, UK) and was incubated for 3 hours at 37°C. After incubation the media was removed and cells washed with phosphate buffered saline. 0.5ml of 10% trichloroacetic acid was then added to each sample for 1 hour to precipitate the DNA. Unbound thymidine was removed by a gentle wash with 4°C phosphate buffered saline. The DNA was then dissolved in 500µl of 0.1% Triton X100 in 10% sodium hydroxide. The final lysates were transferred to a scintillation vial with 10ml of Ecosint H (National Diagnostics, Hull, UK) for counting.

**Real-time quantitative PCR**

Cell RNA was extracted using Trizol reagent and treated with DNase to remove genomic DNA. RNA was then reverse-transcribed to cDNA using M-MLV Reserve Transcriptase. The GeneAmp 5700 sequence detection system (Applied Biosystems, UK) for real-time PCR was used to measure the relative level of gene expression quantitatively. Each sample was amplified with primers and probed for the target gene as well as the housekeeping gene, human á-glucuronidase (hGUS). The sequences for target gene were obtained from NCBI, and the primers and probes were designed using Primer Express software (Applied Biosystems), and shown in Table 1. The results were analysed using GeneAmp 5700 software, and the baseline and threshold were set manually.

To determine if glucose levels may affect the expression of the ERs, the effect of 5 and 25mM glucose on the ER mRNA expression was undertaken over 30min, 2hours, 16 hours, 24 hours, 48 hours and 72 hours, and determined by quantitative PCR.

**Statistical analysis**

Statistical analysis was performed on at least three replicates using ANOVA with Tukeys post hoc analysis (SPSS version 11, SPSS UK Ltd, Surrey, UK). Data are expressed as mean ± standard deviation (SD). Values of \( P<0.05 \) were considered statistically significant.

**Results**

**High glucose induced cell apoptosis and cell proliferation inhibition in HАОECs**

HAоЕCs were found to react differently to different glucose concentrations. Compared to 5mM D-glucose group, 25 mM and 40 mM D-glucose significantly increased cell apoptotic level and reduced proliferation level (*\( P<0.05 \)),
but this was not observed with 11.3 mM glucose-treated group. The treatment of 40 mM D-glucose caused further cell damages than 25 mM D-glucose ($P<0.05$). Detailed data were shown in Figure 1A&B.

Elevated apoptotic level from 0.597±0.005 to 0.430±0.004, reducing 33.5% of induced apoptosis. The apoptotic level in 100 nM genistein-treated cells was even lower than 10 nM genistein group. The $AOD$ value of this group was only 0.297±0.003, and about 60.1% of apoptosis was suppressed. 1 nM genistein did not reduce the apoptotic level caused by high glucose. Similar results were found with cell proliferation detected by $[3H]$-TdR incorporation assay. 17 $β$-estradiol was also found to have protective effects against high glucose-induced cell damages in HAoECs.

**Estrogen receptor expression in HAoECs**

Quantitative PCR showed that only ER$α$ and not ER$β$ or the soluble type of ER$β$ ER$β46$, was expressed in this cell model (Figure 2A). The time course of 5mM and 25mM glucose showed no effect on the expression of the ER$α$ mRNA over 30min to 72 hours (Figure 2B).

**Anti-estrogen ICI182,780 completely blocked the protective effects of genistein against high glucose in HAoECs**

Table 3 showed that cells treated with genistein or 17 $β$-estradiol had significantly higher proliferation level compared to the high glucose group ($P<0.05$), as also shown in Table 2. The co-treatment of ICI182, 780 significantly reduced the cell proliferation to the level comparable to high glucose-treated group. Similar results were obtained from apoptosis detection that genistein and 17 $β$-estradiol re-

![Figure 1](image)

**Figure 1** High glucose altered cell proliferation and cell death in HAoECs

**Genistein reversed high glucose-induced cell apoptosis and proliferation inhibition in HAoECs**

Table 2 showed that genistein at physiological concentrations was able to reduce high glucose-triggered apoptosis and proliferation inhibition in HAoECs. The apoptotic DNA fragmentation level in 25mM D-glucose treated group was significantly higher than the normal control ($P<0.05$). Genistein at 50 nM significantly reduced the elevated apoptotic level from 0.597±0.005 to 0.430±0.004, reducing 33.5% of induced apoptosis. The apoptotic level in 100 nM genistein-treated cells was even lower than 10 nM genistein group. The $AOD$ value of this group was only 0.297±0.003, and about 60.1% of apoptosis was suppressed. 1 nM genistein did not reduce the apoptotic level caused by high glucose. Similar results were found with cell proliferation detected by $[3H]$-TdR incorporation assay. 17 $β$-estradiol was also found to have protective effects against high glucose-induced cell damages in HAoECs.

**Table 2** Effects of genistein and 17β-estradiol on high glucose-induced HAoECs apoptosis and proliferation inhibition

<table>
<thead>
<tr>
<th></th>
<th>ELISA assay</th>
<th>[3H]-TdR assay</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OD value</td>
<td>P value</td>
</tr>
<tr>
<td>Contro</td>
<td>10.098±0.003</td>
<td></td>
</tr>
<tr>
<td>25mM D-glucose</td>
<td>0.597±0.005</td>
<td>$&lt;0.05^*$</td>
</tr>
<tr>
<td>1nM genistein</td>
<td>0.589±0.006</td>
<td>$&gt;0.05^*$</td>
</tr>
<tr>
<td>50nM genistein</td>
<td>0.430±0.003</td>
<td>$&lt;0.05^*$</td>
</tr>
<tr>
<td>100nM genistein</td>
<td>0.297±0.003</td>
<td>$&lt;0.05^*$</td>
</tr>
<tr>
<td>1nM 17$β$-estradiol</td>
<td>0.306±0.004</td>
<td>$&lt;0.05^*$</td>
</tr>
</tbody>
</table>

*compared with the control detected by the same method;

*compared with the value of 25mM D-glucose group detected by the same method; compared to 50nM genistein group detected by the same method
duced apoptotic level, but with the co-treatment of ICI182, 780, the apoptotic levels were elevated back to the level similar to high glucose group (Table 3). The anti-oestrogen ICI182, 780 alone did not cause reduced cell proliferation or increased apoptosis (data not shown).

Discussion

Data here demonstrated the deleterious effect of high glucose on endothelial proliferation and survival. Clinically, hyperglycaemia and oxidative stress are well-recognized risk factors for atherosclerotic diseases, especially the cardiovascular complications in diabetes mellitus. Due to the location among all the constituents in the vessel wall, endothelial cells might be the first to be affected by high glucose concentrations or oxidative stress, thus, endothelial dysfunction in diabetes might play a vital role in the progress of diabetic complications. The inhibited proliferation and apoptotic cell death induced by high glucose exposure and/or oxidative stress might result in an \textit{in vivo} loss of integrity in the endothelial monolayer of blood vessels, leaving the underlined basement membrane exposed to blood ingredients including platelets which aggregated to form thrombi and secreted various growth factors to trigger smooth muscle cell proliferation. It was also demonstrated that oxidative stress- or high glucose-induced endothelial damages included reduced product of nitric oxide\cite{12,13} and overexpression of adhesion molecules\cite{14,15} which facilitated leukocyte/monocyte-endothelium interaction and migration of leukocytes/macrophages into the sub-endothelial layer of blood vessels. These, together with the impaired lipid profile in blood formed the base of atherosclerotic diseases in diabetes.

The soy isoflavones, genistein (5,7,4\textprime; -trihydroxyisoflavone) is the representative phytoestrogen that functions as a putative protective agent against cancer, cardiovascular disease, and osteoporosis. The beneficial effects of phytoestrogen on cardiovascular protection have been indicated by the relatively lower incidence of cardiovascular diseases in people with higher phytoestrogen consumption\cite{5}. In this study, genistein significantly inhibited high glucose-induced cell apoptosis, indicating that they had protective effects against high glucose induced oxidative cell damages in cultured human aortic endothelial cells. This is in accord with reports in literature showing the protective effect of genistein on free radical-induced oxidative damage in other cell types like MC3T3-E1 osteoblast-like cells or human lymphocytes\cite{8}. The results from proliferation assay mirrored the effects on apoptosis, with genistein protecting against the reduction in proliferation from high glucose-induced oxidative stress. 17\textbeta{} -estradiol also showed protective effect in our system, but at much lower doses, the reason for which might be attributed to the different binding affinities among these agents.

The protective effects of genistein and 17\textbeta{} -estradiol were reversed by the pure antiestrogen ICI182, 780 indicating their protective effects being mediated through the estrogen receptor alone, and that no additional mechanism of action through an alternative pathway was occurring. The anti-estrogen ICI182, 780 is commonly used to block the activation of ER\cite{16}. Our findings are consistent with the literature, and it has been reported that the anti-apoptotic

\begin{table}[h]
\centering
\caption{Anti-estrogen ICI182,780 blocked the effects of genistein and 17\textbeta{}-estradiol on high glucose-induced cell damage in HAoECs}
\begin{tabular}{|c|c|c|}
\hline
 & \textbf{ELISA assay} & \textbf{[\textit{3}H]-TdR assay} \\
 & \textbf{OD value} & \textbf{P value} & \textbf{\textit{\textalpha{}}-reading} & \textbf{P value} \\
\hline
Normal Control & 0.098\pm0.003 & --- & 3450.71\pm30.31 & --- \\
25mM D-glucose & 0.597\pm0.005 & <0.05 & 2146.63\pm27.38 & <0.05 \\
100nM genistein & 0.297\pm0.003 & <0.05* & 2792.15\pm30.17 & <0.05* \\
+100nM AE & 0.589\pm0.002 & >0.05* & 2152.47\pm29.44 & >0.05* \\
1nM 17\textbeta{}-estradiol & 0.306\pm0.004 & <0.05* & 2812.36\pm25.74 & <0.05* \\
+100nM AE & 0.592\pm0.004 & >0.05* & 2212.76\pm31.33 & >0.05* \\
\hline
\end{tabular}
\end{table}

\*compared with 25mM D-glucose group detected by the same method; AE: anti-estrogen ICI182,780

\textbf{Figure 2 Estrogen receptor b expression in HAoECs}

A. Estrogen receptor b was found to be expressed in HAoECs - hGUS; - ERb
B. 25mM D-glucose did not change the expression level of ERb over 72 hours incubation

\textbf{Table 3}
effect of genistein in neurons was ER dependent and was reversed by anti-estrogen co-treatment[16]. The mRNA of ERα but not ERβ was found to be expressed in HAoECs in our system. Whether this is due to the techniques employed or the culture conditions used it is unclear why ERs have been found by some but not by others. There was not direct effect of glucose on the expression of ER mRNA, which suggested that high glucose is having an effect downstream of transcription, perhaps at the level of translation or ER cofactors.

In conclusion, isoflavone genistein protected against high glucose-induced cell apoptosis and proliferation inhibition in human aortic endothelial cells that express ERα, by an estrogen dependent mechanism, and these effects are completely reversed by the antiestrogen ICI182,780. High glucose was likely to have an effect downstream of ER transcription, which needs to be investigated in the future work. Hypothetically, the anti-oxidative properties against high glucose of isoflavone genistein may give clinical arterial endothelial cell protection through the activation of estrogen receptor, by which the proliferative activity, viability and even some active substances-secreting ability may be restored at the condition of diabetes or hyperglycemia, and therefore, help delay the onset and development of cardiovascular complications.

References

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