



REGULAR ARTICLE

# The dietary soy flavonoid genistein abrogates tissue factor induction in endothelial cells induced by the atherogenic oxidized phospholipid oxPAPC

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## KEYWORDS

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## Abstract

**Introduction:** Tissue factor (TF) plays a pivotal role in the generation of thrombin in atherothrombotic disease. The oxidized phospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (oxPAPC), an active compound of minimally oxidized low-density lipoprotein (MM-LDL), induces TF in endothelial cells (EC). The dietary soybean isoflavonoid genistein has been claimed to reverse several processes leading to atherosclerosis and related cardiovascular events via binding to estrogen receptors, generating nitric oxide (NO) or inhibiting tyrosine kinase-dependent pathways.

**Methods and materials:** The effects and mechanisms of genistein on activity, antigen expression and mRNA levels of oxPAPC-induced TF were studied in human umbilical vein endothelial cells (HUVEC) and human aortic endothelial cells (HAEC).

**Results and conclusions:** Genistein abrogated oxPAPC-induced TF activity in arterial and venous human EC to basal levels, as measured by functional clotting assay, and

*Abbreviations:* oxPAPC, oxidized phospholipid 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine; MM-LDL, minimally oxidized low density lipoproteins; EC, endothelial cells; L-NAME, (omega)-nitro-L-arginine methyl ester; NO, nitric oxide.

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downregulated oxPAPC-induced antigen expression measured by flow cytometry and mRNA levels quantified by real-time PCR. Western blotting and inhibitor experiments with the estrogen-receptor inhibitor ICI 182,780 and the NO-synthase inhibitor *N* (omega)-nitro-L-arginine methyl ester (L-NAME) showed that the effect may be mediated via inhibition of phosphorylation of ERK, but not upstream MEK1/2. The effect is not mediated by the tyrosine kinase inhibitor activity of genistein, as another tyrosine kinase inhibitor (tyrphostin 25) had no effect. Binding to the estrogen receptor or generation of NO are not involved in the action of genistein on TF. In conclusion genistein reduces oxPAPC-induced TF expression and thereby the prothrombotic phenotype of EC, further substantiating and explaining the beneficial effects of dietary genistein in preventing atherosclerosis and related cardiovascular events.

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## Introduction

Tissue factor (TF) is the predominant activator of blood coagulation. It interacts with coagulation factor VII/VIIa and initiates blood coagulation when the integrity of the vasculature is corrupted or endothelial cells are induced to express cell surface TF by various stimuli [1,2]. TF expression on the cell surface is associated with thromboembolic complications in inflammation [3], sepsis [4], and atherosclerotic disease [5]. On the atherosclerotic plaque the surface expression of TF plays a major role in determining the plaque's thrombogenicity [6]. Oxidized phospholipids are thought to contribute to atherogenesis [7]. For example, oxidized phospholipids derived from 1-palmitoyl-2-arachidonoyl-*sn*-glycero-3-phosphorylcholine (oxPAPC) have been characterized [8] and demonstrated to be biologically active compounds in minimally modified LDL (MM-LDL) [9]. They have been associated with atherogenesis and cardiovascular disease (CVD) through several cellular processes [7], e.g. the induction of TF in endothelial cells (EC) [10]. Emerging data indicate that high dietary intake of soy isoflavones through soy protein rich diets might exert beneficial effects on the cardiovascular system [11,12], but this also has been questioned [13]. Particularly the isoflavonoid genistein, a polyphenolic non steroidal flavonoid, which can be found in abundance in soybeans, has drawn a lot of attention because of its structure being similar to that of 17- $\beta$  estradiol and its strong estrogenic properties [14].

Genistein belongs to the group of phytoestrogens and binds to the alpha and beta estrogen (ER) receptors [15]. Genistein at concentrations in the  $\mu$ M range has also been shown to alter the phosphorylation of intracellular proteins by inhibiting tyrosine specific kinases [16] and to induce NO-synthesis [17,18]. Studies have been conducted

investigating the cardiovascular effects of human and animal dietary supplementation with genistein. In a myocardial ischemia and reperfusion injury rat model [19], diet supplementation with genistein has proven to act in a cardioprotective and anti-inflammatory way. In other studies it has been shown to improve resistance of LDL against oxidation *ex vivo* [20] and systemic arterial compliance in (peri)menopausal women [21]. Further, genistein has been demonstrated to ameliorate endothelial dysfunction in postmenopausal women by improving flow mediated endothelium-dependent dilatation and increasing the ratio of nitric oxide to endothelin, which is a parameter for endothelial function [22].

Experimental studies have addressed different cellular effects of genistein *in vitro* which might contribute to its cardiovascular effects observed *in vivo*: Genistein has been demonstrated to inhibit LDL-oxidation and to protect cells against the damage of oxidized lipids [23]. It has also been shown to inhibit proliferation and angiogenesis in vascular endothelial cells [24]. In smooth muscle cells genistein inhibits proliferation and chemotaxis [25], in platelets it has been demonstrated to antagonize activation [26] and procoagulant activity [27].

With regard to the reported effects of genistein *in vivo* and *in vitro* and the importance of TF in atherosclerotic and cardiovascular disease we tested the hypothesis that genistein will antagonize expression of oxPAPC-induced TF in endothelial cells.

## Materials and methods

All cell culture media and M-MLV reverse transcriptase were obtained from Invitrogen. Endothelial cell growth supplement isolated from calf hypothalamus was kindly provided by J. Wojta (Medical

University Vienna). PAPC, genistein, genistin, daidzein, tyrphostin 25, rabbit brain thromboplastin, L-NAME and collagenase were from Sigma. Random hexamers and ECL reagents were purchased from Amersham Biosciences. Capillaries, Fast Start DNA Master SYBR Green I kit and calcium chloride were from Roche Diagnostics. FITC labeled monoclonal antibody (mAb) against TF was obtained from American Diagnostica Inc., PhosphoSafe™ extraction buffer (including the phosphatase inhibitors sodium fluoride, sodium vanadate,  $\beta$ -glycerolphosphate and sodium pyrophosphate) was from Novagen. Rabbit polyclonal anti-phospho ERK 1/2 (Thr177) Ab was from Santa Cruz, polyclonal rabbit anti-phospho MEK1/2 (Ser217/221) Ab was from Cell Signaling, mouse anti  $\beta$ -actin mAb was from Abcam. Secondary antibodies (ECL anti-rabbit and anti-mouse IgG, peroxidase-linked) were obtained from Amersham Biosciences. Combi Reagent negative control was purchased from An der Grub, RNAzol B reagent from TEL-Test, and ICI 182,780 from Tocris Cookson Inc.

## Cell culture

Cultures of human umbilical vein endothelial cells (HUVEC) were established as described by Jaffe et al. [28]. On day 2 after isolation medium was removed and cells were grown in RPMI-1640 medium supplemented with 5% FBS. Medium was changed every second day. After the first passage cells were grown in M-199 with 20% FCS including ECGS and heparin. For experiments passage 3-5 HUVEC were grown to confluence in six-well plates. For the isolation of human aortic endothelial cells (HAEC), aortic tissue from organ-donors was incubated in 20 mmol L<sup>-1</sup> phosphate-buffered saline containing 50 mg of collagenase H/mL for 30 min at 37 °C. Cells were passed through a 100  $\mu$ m mesh and plated on petri dishes in EGM-2MV microvascular endothelial-cell medium. After 12 h, the nonadherent cells were removed, and fresh medium was added to the adherent cells. The medium was changed every 3 days. After 5 to 10 days, the adherent cells were detached from the petri dish by incubation with 0.25% trypsin and 1 mM L<sup>-1</sup> EDTA and incubated with magnetic beads that were coated with CD31 antibody (Dyna), according to the manufacturer's instructions. Microbeads with bound cells were replated on petri dishes. The beading procedure was repeated after another 5 to 10 days of culture. Passage 3-8 HAECs were used for experiments. Before stimulation, cells were exposed for 2 h to RPMI-1640 medium without phenol red containing 100 U mL<sup>-1</sup> penicillin, 100  $\mu$ l mL<sup>-1</sup> streptomycin, 5  $\mu$ g mL<sup>-1</sup> fungizone and 1% L-glutamine with 5% FCS

or without FCS (for Western blot analyses). Stimulation were conducted with the same medium containing mediators for 6 h for the TF activity assay, the surface antigen assay and the tissue factor pathway inhibitor (TFPI) antigen assay, for 3 h for the TF real-time RT-PCR, and for 5 to 15 min for Western blot analysis of p-ERK 1/2 and p-MEK 1/2. Stock solutions (5 mmol L<sup>-1</sup>) of genistein, genistin, daidzein and tyrphostin 25 were prepared in 0.1 mol L<sup>-1</sup> Na<sub>2</sub>CO<sub>3</sub>, which were stored at 4 °C and used for 2 weeks.

## Lipid oxidation

PAPC was oxidized by exposure of dry lipid to air for 72 h. The extent of oxidation was monitored by positive ion electron spray mass spectrometry as described previously [8].

oxPAPC contained less than 50 pg mL<sup>-1</sup> of endotoxin as determined by the Limulus ameocyte assay (Bio Whittaker).

## Tissue factor activity assay

TF assay for the quantification of the procoagulant activity of endothelial cells was performed as previously described [29]. After incubations, cells were scrape-harvested and washed with PBS. Cells suspended in 500  $\mu$ l PBS were sonicated for 15 s at 4 °C. The cell lysate was then assayed in a one stage clotting assay for procoagulant activity: 50  $\mu$ l citrated normal donor platelet-poor plasma was incubated for 1 min with 50  $\mu$ l of cell lysate at 37 °C in prewarmed plastic tubes of a ST-Art coagulometer (Stago); 50  $\mu$ l CaCl<sub>2</sub> (30 mmol/l) was then added and the coagulation time was measured. Control experiments were performed with factor VII-(Sigma), IX-(Technoclone), and X (Biopool)-deficient plasmas to characterize the procoagulant activity measured as TF activity. TF activity equivalents were determined from a standard curve obtained using rabbit brain thromboplastin.

## Tissue factor surface expression on EC

To evaluate EC-surface expression of TF after exposure to different mediators, cells were analyzed by flow cytometry. Confluent EC were detached by use of a collagenase-treatment (type Ia, 200 U mL<sup>-1</sup> final concentration) for 10 min, washed twice in ice-cold PBS, and exposed to FITC labeled mAb directed against TF for 30 min at 4 °C. Then, cells were washed two times in ice-cold PBS and analyzed. The reactivity of the antibodies with EC was determined by flow cytometry (FACScan,

Becton Dickinson). Irrelevant IgG<sub>1</sub>+IgG<sub>2a</sub> was used as negative control. Experiments were done twice.

### Tissue factor pathway inhibitor (TFPI) antigen in EC

TFPI antigen levels in HUVEC were measured after 6 h of incubation in HUVEC supernatants and extracts by ELISA using a commercially available kit (Imubind TFPI, American Diagnostica) according to the manufacturer's instructions. After collection of supernatants (which were frozen at  $-70^{\circ}\text{C}$  until measurement), EC were washed twice with PBS and then extracted in Tris buffer ( $5 \times 10^5$  cells/mL of 50 mmol/L Tris buffer, 100 mmol/L NaCl, 0.1% Triton X-100, pH 7.4) for 18 h at  $4^{\circ}\text{C}$  and frozen at  $-70^{\circ}\text{C}$  until measurement.

### RNA-isolation and cDNA preparation

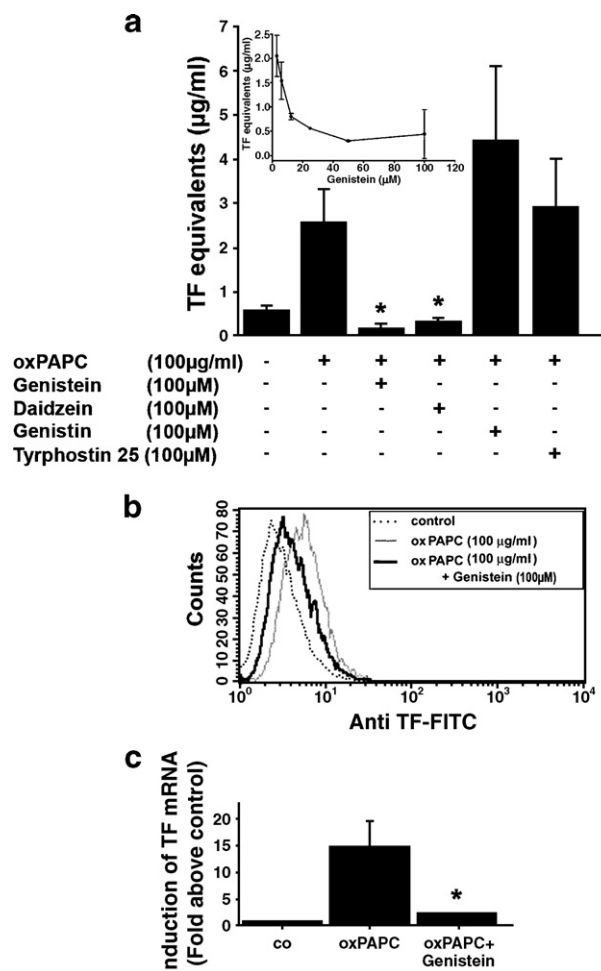
RNA was isolated from HUVEC using RNAzol B reagent according to the protocols of the manufacturers. 1  $\mu\text{g}$  of total RNA was reverse transcribed with M-MLV-RT using random hexamer primers.

### Quantitative RT-PCR

Human TF primers were designed using PRIMER 3 software from the Whitehead Institute for Biomedical Research [30]. The amplified cDNA regions were chosen to span one or more large introns in the genomic sequence, thus avoiding co-amplification of genomic DNA. The testing of primer specificity included melting curve analyses, agarose gel electrophoresis of the PCR products, and subsequent DNA sequencing. Quantitative RT-PCR was performed by Light Cycler (Roche Diagnostics) using SYBR Green I detection. In all assays, cDNA was amplified using a standardized program: (10 min denaturing step; 55 cycles of 5 s at  $95^{\circ}\text{C}$ , 5 s at  $65^{\circ}\text{C}$  and 15 s at  $72^{\circ}\text{C}$ ; melting curve analysis in  $0.1^{\circ}\text{C}$  steps). Light Cycler capillaries were each loaded with a final volume of 15.3  $\mu\text{l}$  (1.5  $\mu\text{l}$  DNA Master Mix, 1.8  $\mu\text{l}$   $\text{MgCl}_2$  ( $25 \text{ mM L}^{-1}$ ), 10.1  $\mu\text{l}$   $\text{H}_2\text{O}$ , 0.4  $\mu\text{l}$  of each primer ( $10 \mu\text{mol L}^{-1}$ ) and 1.5  $\mu\text{l}$  cDNA). The final amount of cDNA corresponded to 19.8 ng of total RNA used for reverse transcription. Relative quantification of target gene expression was performed according to recommended standard procedures. The following primers were used: TF (Hs TF, NM 001993, [31]): CCGAACAGTTAACCGGAAGA r: TCAGTGGGGAGTTCTCCTTC (RT-PCR efficiency 1.749).  $\beta$ -actin: (Hs ACTB, NM 001101): f CGCGAGAAGACCCAGATC r: TCACCGGAGTCCATCACGA (RT-PCR efficiency 2.01).

### Western blot analysis

Confluent EC monolayers were exposed to oxPAPC ( $100 \mu\text{g mL}^{-1}$ ) with and without genistein



**Figure 1** Genistein reduces oxPAPC-induced tissue factor in HUVEC. oxPAPC induces TF activity, while genistein reduces oxPAPC-induced TF activity (a) in a concentration-dependent manner (insert), protein expression (b) and mRNA levels (c) in HUVEC. The genistein analogue daidzein, but not genistein or tyrphostin 25 ( $100 \mu\text{mol L}^{-1}$  each) also reduced oxPAPC-induced TF activity (a). HUVEC were treated with oxPAPC ( $100 \mu\text{g mL}^{-1}$ ) for 6 (a, insert, b) or 3 (c) h with or without genistein ( $100 \mu\text{mol L}^{-1}$ , concentration dependency in insert). oxPAPC-induced TF activity and TF antigen, as well as TF mRNA levels were measured as described in Materials and methods. oxPAPC-induced TF activity (a, insert) is expressed in TF activity equivalents, as described in Materials and methods. Data are representative of four independent experiments and expressed as mean  $\pm$  S.D. TF surface antigen is expressed as mean fluorescence intensity, the plot shown represents one of two independent experiments. mRNA levels were calculated as relative expression as described in Materials and methods, the data represent the mean  $\pm$  S.D. relative expression of three independent experiments. \* $p < 0.05$ , compared with oxPAPC alone.

(100  $\mu\text{mol L}^{-1}$ ) for 5 to 15 min. Thereafter, supernatant was removed and cells were lysed in 50  $\mu\text{l}$  of PhosphoSafe™ buffer per 35 mm well. 40  $\mu\text{g}$  protein per lane was separated on a 10% SDS-polyacrylamide gel and transferred to nitrocellulose membranes. After blocking membranes with blocking buffer (dry milk dissolved in PBS), anti p-ERK1/2 (1:1000), anti p-MEK 1/2 (1:1000) and anti  $\beta$ -actin (1:15,000) antibodies were added in 5% BSA, 1 $\times$  TBS, 0.1% Tween-10 at 4  $^{\circ}\text{C}$  overnight. Bound primary antibody was detected by anti-IgG conjugated with peroxidase and subsequent chemoluminescent (ECL, Amersham Biosciences) detection. Experiments were done twice.

## Statistics

Data are presented as means  $\pm$  S.D. (at least  $n=4$  for TF activity and  $n=3$  for mRNA analyses). Wilcoxon rank-sum test was used for calculating statistically significant differences. A statistically significant difference is achieved if the difference from the null hypothesis reaches  $p<0.05$ . All statistical calculations were made with SPSS 10.0 for Windows.

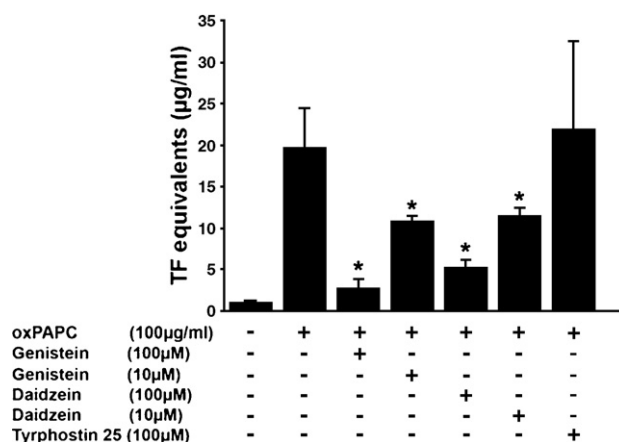
## Results

### Genistein abrogates oxPAPC-induced EC TF

oxPAPC can enhance procoagulant activity of HUVEC, as demonstrated before [10]. We first determined dose dependency and time course curves to define maximum effects of oxPAPC. The TF-inducing effect of oxPAPC proved to be con-

centration- and time-dependent with maximal effects around 100  $\mu\text{g mL}^{-1}$  at 6 h (not shown). 100  $\mu\text{g mL}^{-1}$  oxPAPC has been previously proven not to be toxic to HUVEC [10]. Thus, 100  $\mu\text{g mL}^{-1}$  oxPAPC was used to induce TF for all subsequent experiments. After 6 h of incubation with oxPAPC HUVEC demonstrated higher cellular TF activity in the clotting assay and higher cell surface expression of TF protein in the flow cytometry assay in comparison to unstimulated controls (Fig. 1a, b). In concordance with these results, TF specific mRNA levels were higher in oxPAPC stimulated HUVEC than in unstimulated controls, as measured by RT-PCR after an incubation time of 3 h (Fig. 1c). Co-incubation of the soy isoflavonoid genistein with oxPAPC for 6 h significantly decreased TF activity of HUVEC in comparison to cells which were incubated with oxPAPC only (Fig. 1); this effect was concentration dependent (Fig. 1a insert). The abrogation of oxPAPC-induced TF activity was also paralleled by surface TF protein expression (Fig. 1b) and TF-mRNA expression (Fig. 1c). The genistein analogue daidzein was as effective as genistein in antagonizing TF activity (Fig. 1a). In contrast, genistin, the 7-O-glucoside of genistein was not active in this respect (Fig. 1a). To test, if the effect was dependent on the well known ability of genistein to inhibit tyrosine kinases, the specific protein tyrosine kinase inhibitor tyrphostin 25 - which has no polyphenolic structure in contrast to genistein - was tested. Tyrphostin 25 could not mimic the effect of genistein on TF (Figs. 1a and 2).

With respect to our specific hypothesis, we confirmed the key data with arterial endothelial



**Figure 2** Genistein reduces oxPAPC-induced tissue factor in HAEC. HAEC were treated with oxPAPC (100  $\mu\text{g mL}^{-1}$ ) for 6 h with or without genistein, daidzein (100  $\mu\text{mol L}^{-1}$  and 10  $\mu\text{mol L}^{-1}$  each) or tyrphostin 25 (100  $\mu\text{mol L}^{-1}$ ). Genistein and daidzein, but not tyrphostin 25 antagonized oxPAPC induced TF activity. oxPAPC-induced TF activity is expressed in TF activity equivalents, as described in Materials and methods. Data are representative of two independent experiments in triplicates and expressed as mean  $\pm$  S.D. \* $p<0.05$ , compared with oxPAPC alone.

cells from human aorta. As shown in Fig. 2, daidzein abolished oxPAPC induced TF expression also in HAEC, whereas genistein and tyrphostin 25 was inactive.

### The effect of genistein on EC TF is not mediated by increased EC TFPI

To test, if genistein affects EC TF expression via increased TFPI secretion, HUVEC were incubated for 6 h with genistein, genistin or daidzein (100  $\mu\text{mol L}^{-1}$  each). No induction of secretion of TFPI into the medium was seen with any of these treatments (control:  $0.87 \pm 0.66$  ng/mL, genistein:  $0.54 \pm 0.45$  ng/mL, genistin:  $0.70 \pm 0.54$  ng/mL, daidzein:  $0.61 \pm 0.48$  ng/mL;  $n=7$ ). TFPI was also not induced by either of these treatments in HUVEC lysates (not shown).

### Genistein does not reverse upregulation of TF-activity by oxPAPC via the estrogen receptor

As a phytoestrogen genistein binds to the estrogen receptors alpha and beta and might exert some of its postulated actions via this receptor. Addition of the highly selective estrogen receptor antagonist ICI 182,780 did not alter the observed attenuation of TF-activity of HUVEC being co-incubated with oxPAPC and genistein compared to HUVEC being incubated with oxPAPC only (Fig. 3). Moreover, we found no difference in the effects of genistein on oxPAPC-induced TF expression in HUVEC obtained from female (which were demonstrated to have increased expression of ER alpha and ER beta

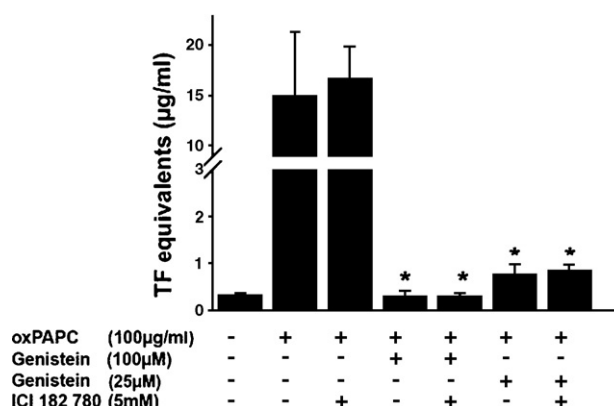
mRNA in contrast to those obtained from male newborns [32] versus male newborns (data not shown).

### Nitric oxide (NO) plays no intermediate role in downregulating TF in oxPAPC stimulated HUVEC

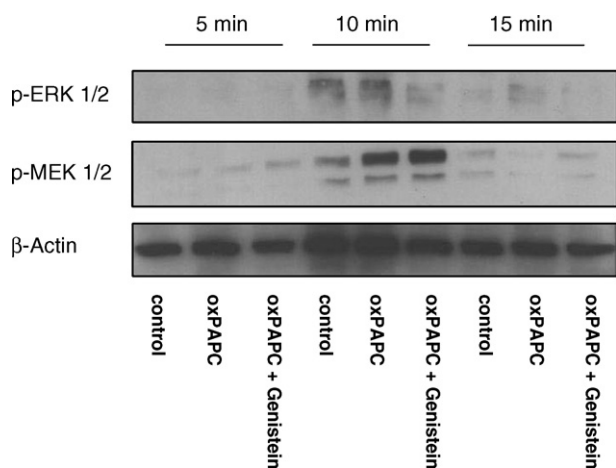
Suppression of NO synthesis has been associated with increased expression of TF in endothelial cells [33], while genistein in the  $\mu\text{M}$ -range has been claimed to increase NO synthesis in these cells [17,18]. To determine whether genistein induced NO could also reverse oxPAPC induced TF-expression, we tested the effects of genistein on oxPAPC-stimulated HUVEC in the presence of an inhibitor of NO-synthesis, L-NAME, in a concentration (300  $\mu\text{mol L}^{-1}$ ) which has been demonstrated before [32] to attenuate significantly basal- and agonist-stimulated NO-release by endothelial cells. Genistein (100 and 50  $\mu\text{mol L}^{-1}$ ) attenuated TF-expression induced by oxPAPC. Addition of L-NAME did not exert any influence on this effect (data not shown).

### Genistein inhibits oxPAPC induced phosphorylation of ERK 1/2, but not MEK 1/2

To test, if genistein exerts its effects via inhibition of the MEK/ERK pathway, which is involved in oxPAPC-induced TF induction [10], we looked for phosphorylation of ERK1/2 and MEK1/2 in Western blot experiments. oxPAPC-induced phosphorylation of the protein kinases MEK1/2 and downstream ERK1/2 in EC occurred within 5 to 15 min of



**Figure 3** The effects of genistein on oxPAPC-induced TF induction are not mediated via estrogen receptors on HUVEC. HUVEC were treated with oxPAPC (100  $\mu\text{g mL}^{-1}$ ) for 6 h with or without genistein (100  $\mu\text{mol L}^{-1}$  or 25  $\mu\text{mol L}^{-1}$ ). The estrogen receptor antagonist ICI 182,780 was added 1 h before and during genistein incubations. Genistein abolished oxPAPC-induced TF activity. This effect was not altered by ICI 182,780 treatment. oxPAPC-induced TF activity is expressed in TF activity equivalents, as described in Materials and methods. Data are representative of two independent experiments in triplicates and expressed as mean  $\pm$  S.D. \* $p < 0.05$ , compared with oxPAPC and genistein.



**Figure 4** Genistein inhibits oxPAPC induced phosphorylation of ERK 1/2, but not MEK1/2. Representative picture of a Western blot analysis (out of two experiments done) demonstrating the effect of genistein on oxPAPC induced phosphorylation of ERK1/2 and MEK1/2 in HUVEC. HUVEC were incubated with oxPAPC ( $100 \mu\text{g mL}^{-1}$ ) or oxPAPC ( $100 \mu\text{g mL}^{-1}$ ) and genistein ( $100 \mu\text{mol L}^{-1}$ ) for 0, 5 and 15 min. Cell lysates were separated on a 10% polyacrylamide gel and phosphorylated proteins were detected using specific antibodies as described in Materials and methods. oxPAPC induced phosphorylation of ERK1/2 and MEK1/2. Phosphorylation of ERK1/2, but not MEK1/2 was antagonized by genistein at 10 and 15 min.

oxPAPC treatment (Fig. 4). The addition of genistein to ox-PAPC stimulated HUVEC abolished the phosphorylation of ERK1/2, but not of MEK1/2 (Fig. 4).

## Discussion

TF, the key initiator of the coagulation cascade, is expressed in EC only after stimulation. Cytokines, such as  $\text{TNF}\alpha$ , IL-1 $\beta$  or CD40 ligand, biogenic amines such as serotonin and histamine, vascular endothelial growth factor, thrombin and oxidized lipids can induce TF in EC. Despite their diversity, most inducers share signal transduction pathways (i.e. the ERK pathway) regulating TF induction [34].

In this study we have shown that genistein exerts anticoagulant effects by downregulating oxPAPC-induced TF activity, surface protein expression and mRNA levels in EC, counteracting the procoagulant effects of oxPAPC ( $100 \mu\text{g mL}^{-1}$ ), an oxidized phospholipid and active compound of MM-LDL [8] which has been implicated to play a major role in the development of atherosclerosis [7].

Data from epidemiological and clinical studies have promoted the idea that high dietary intake of soy protein might have a beneficial impact on cardiovascular health [12]. Some researchers have

claimed that this effect might be attributable to the soy isoflavonoid genistein which is abundant in soy protein [19,20,22] and results from *in vitro* studies have addressed the cellular effects of genistein, explaining its potential cardioprotective properties [23,35,36]. Here we demonstrate that genistein is able to attenuate TF activity of arterial and venous EC which have been stimulated with oxPAPC. These results were paralleled by a decrease in TF cell surface protein and TF mRNA levels. The TF-activity suppressive effect of genistein was concentration-dependent. Dosages in the low  $\mu\text{M}$ -range, which were effective in our experiments, have been demonstrated before to correspond to genistein blood levels in persons consuming soy supplements or a soy-rich diet [37]. In experiments with the soy-derived genistein analogue daidzein, which also possesses a polyphenol structure, a similar effect on TF activity could be observed, whereas the 7-O-glucoside genistin did not act in this manner. This might be due to the loss of the polyphenolic character by the O-glucoside formation. The specificity of the effect was substantiated by experiments with the non soy-derived specific protein tyrosine kinase inhibitor tyrphostin 25, which did not mimic the effect of genistein in reducing oxPAPC induced TF activity. As tyrphostin 25 as well as genistein is a specific tyrosine kinase inhibitor, this argues against an unspecific effect mediated by an upstream signaling event involving tyrosine phosphorylation.

The extent of TF protein induction in vascular cells does not always correlate well with TF activity. One explanation is the concomitant secretion of TFPI [34]. In this study, the effect of genistein was not mediated by an induction of EC TFPI production, as TFPI levels after 6 h of incubation of HUVEC with genistein, genistin or daidzein did neither induce increased secretion of TFPI into the supernatants, nor increased TFPI in HUVEC lysates.

The estrogen 17 $\beta$ -estradiol has been demonstrated before to downregulate TF protein and mRNA in HUVEC [38]. As a phytoestrogen genistein binds to estrogen receptors [14,15] and can thereby mimic estrogenic effects or inhibit them. Thus it would have been reasonable if the TF-attenuating effects of the isoflavonoid on oxPAPC stimulated EC in our setting could be related to its estrogen-receptor binding capacities. This possibility, however, could be ruled out, as the isoflavonoid's effect on TF expression remained the same in the presence of the estrogen receptor antagonist ICI 182,780. This finding was further substantiated by the fact that daidzein was able to suppress oxPAPC-induced TF expression: Daidzein binds to the estrogen receptor with much lower affinity ( $\text{ER}\alpha$ : 0.1%,  $\text{ER}\beta$ : 0.5% of

estrogen) in comparison to genistein (ER $\alpha$ : 4%, ER $\beta$ : 87% of estrogen) [39], but showed same activity on oxPAPC-induced TF expression in our experiments. Finally, the effect of genistein was not different in EC of female vs. male origin. As female EC express higher levels of estrogen receptors [32], this also argues against an ER-receptor mediated process. All together, the absence of any estrogen receptor binding activity of genistein in context with our observed anticoagulant effects could be explained by the concentration of the isoflavonoid in our experiments. Effects of genistein on endothelial cells which are conducted via estrogen receptor have been reported, but only in context with lower genistein concentrations [32].

NO has been assigned a major regulatory role in the expression of TF in the endothelial cell [40,41]. While oxidized phospholipids have been demonstrated to be capable to inhibit NO production [42], genistein in dosages of the  $\mu$ M-range has been shown to increase synthesis and release of NO via NOS [17,18] independently of binding to the estrogen receptors or inhibiting tyrosine kinases. In order to investigate whether the genistein induced generation of NO could also antagonize TF expression in oxPAPC-stimulated cells we tested the observed anticoagulant effects of genistein in the presence of an inhibitor of basal and induced NO-synthesis, L-NAME [32]. The addition of L-NAME to wells incubated with oxPAPC and genistein did not alter TF-activity in comparison to cells being incubated with oxPAPC and genistein only. Thus, the anticoagulant effects of genistein on oxPAPC induced TF expression cannot be ascribed to its NO inducing abilities. This is also in accordance with recent reports demonstrating that genistein in the  $\mu$ M range enhances NO synthesis acutely (10-120 min) and late onset (24-48 h) in EC, where oxPAPC induced TF expression reaches its maximal effect after 6 h. Finally, we wanted to investigate if genistein interferes with the ERK/MEK pathway, which has recently been identified to play a major role in oxPAPC-induced TF induction [10]. According to our results genistein interferes with this pathway downstream of MEK1/2 by inhibiting phosphorylation of ERK1/2. The direct inhibition of oxPAPC-induced ERK1/2 phosphorylation by genistein may contribute to the effect of this flavonoid on TF in EC.

In conclusion, our findings substantiate existing *in vivo* data which indicate that high dietary intake of genistein could promote cardiovascular health and we could further demonstrate that these effects may partly be due to a direct, non-estrogenic influence of genistein on the coagulant properties of the endothelium.

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