Dear Sirs,

Plasminogen activator inhibitor 1 (PAI-1) became a molecule of major clinical interest because various diseases like atherosclerosis, thrombosis, myocardial infarction, or type 2 diabetes mellitus were found to be associated with high PAI-1 levels (1–3). PAI-1 gained even more importance since it became validated as a high-level-of-evidence marker for poor prognosis in breast cancer (4, 5). Hypoxia, not only a hallmark of solid tumours but also associated with the above mentioned diseases, is among the major PAI-1 inducers (6).

In recent years resveratrol, a polyphenol found in red grape skin, berries and medicinal plants, has been subject of intense interest since it was not only considered to explain the „French Paradox“ (7) but also to be protective against a number of diseases, e.g. cardiovascular disease, Alzheimer disease, and cancer (8–11).

Given the link between PAI-1, cancer and hypoxia, as well as the potential anti-cancer effect of resveratrol one would expect that resveratrol antagonizes the cancerous hypoxia-induced PAI-1 expression. However, the ongoing debate whether PAI-1 is a “good” or “bad” protein and whether its increased levels contribute to diseases or reflect the repair process initiated by the healthy surrounding cells (12) make it at the moment difficult to predict the outcome of resveratrol treatment. To gain further insight into the action of resveratrol on PAI-1 expression in cancer and non-cancer cells we studied effects of resveratrol under normoxia and hypoxia in the liver tumour cell line HepG2 and in primary hepatocytes (pHC). In line with previous studies (13, 14) both cell types responded to hypoxia with an induction of PAI-1 expression. Surprisingly, upon treatment with resveratrol the two cell types showed a different PAI-1 expression pattern. Treatment of HepG2 cells with resveratrol induced PAI-1 protein levels under normoxia and hypoxia in a time- and concentration-dependent manner; the resveratrol effect was additive to the hypoxia effect. In contrast, in pHC resveratrol did not affect PAI-1 protein levels under normoxia, but reduced hypoxia-dependent PAI-1 induction (Figure 1A, B, C). This pattern was also observed on PAI-1 mRNA levels (Figure 1D). Given that PAI-1 is a well-known target gene of HIF-1α (13, 14) and p53 (15, 16) under different conditions, we analysed HIF-1α and p53 protein levels after the cells were treated with hypoxia, resveratrol or both for 4 hours (h) (data not shown) or 16 h (Figure 1C). The addition of resveratrol did not influence HIF-1α and p53 levels in both cell lines at the two studied time points suggesting that HIF-1α or p53 stabilisation/destabilisation is not involved in mediating the resveratrol effects on PAI-1. Thus, resveratrol appears to act on PAI-1 either in a HIF-1α and p53-independent manner or by changing only their activity which brings an intriguingly new aspect to PAI-1 regulation under different physiological and pathological conditions.

So far, most effects of resveratrol were studied in rodent models and recent reports suggest interspecies differences in PAI-1 transcriptional regulation due to variations in the promoter context (17, 18). To rule out that these are relevant for the opposite effects, both cell types were transfected with either human (pGL3hPAI-806/+19) or rat (pGL3rPAI-766) PAI-1 promoter- luciferase (Luc) constructs and treated with resveratrol under normoxia and hypoxia. While hypoxia activated both promoter constructs in both cell types, the effects of resveratrol were again opposite. When both promoter constructs were transfected into HepG2 cells, treatment with resveratrol resulted in an induction of Luc activity under normoxia and hypoxia. In contrast, resveratrol completely abolished the hypoxia-mediated Luc response of both promoters in primary cells (Figure 1E, F). Together, these data show that the opposite resveratrol effects on PAI-1 are due to the cell systems showing likely differences in HIF activity (not protein levels) rather than on differences in the promoter context.

To the best of our knowledge the present data are first comparing resveratrol effects on PAI-1 expression in tumour vs non-tumour cells under normoxia and hypoxia. Although descriptive, the current data open up new directions in thinking about resveratrol’s biological activities. A huge number of publications considered resveratrol to be “beneficial”, but there are also several challenging-the-concept studies showing that resveratrol is growth promoting in cancer cells (19) and that it negatively affects wound healing (20). With respect to PAI-1 resveratrol was shown to be rather an inhibitor than an inducer. Intriguingly, these studies show that this occurs in non-cancerous tissue explants or non-cancer cells like in this study. Moreover, the inhibitory effect of resveratrol on PAI-1 is much stronger in cells under stress.

Correspondence to:
Dr. Elitsa Dimova
Faculty of Biochemistry and Molecular Medicine
University of Oulu
Aapistie 7B
90230 Oulu, Finland
Fax: +358 8 553 1141
E-mail: Elitsa.Dimova@oulu.fi

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Figure 1: Resveratrol exerts opposite effects on PAI-1 expression in the human liver hepatoma cell line HepG2 and primary hepatocytes (pHC). HepG2 cells (in MEM supplemented with 10% FCS and 1% non-essential amino acids) and pHC (in M199 containing 0.5 nmol/l insulin, 100 nmol/l dexamethasone, and 4% FCS for the initial 5 h of culture) were cultured under normoxia (16% O₂) for 24 h. At 24 h the medium was changed and the cells were further cultured for the indicated times and concentrations of resveratrol (Res) under normoxia and hypoxia (5% O₂); controls were treated with DMSO. In all experiments PAI-1 readouts under normoxia were set to 100% or to 1. Values represent means ± SEM of at least three independent experiments. Statistics, Student t-test for paired values: * significant difference 16% O₂ vs 5% O₂, ** significant difference 16% O₂ vs 16% O₂ + Res and 5% O₂ vs 5% O₂ + Res; p ≤ 0.05.

A, B, C) Time- and concentration-dependent regulation of PAI-1 protein levels by resveratrol in HepG2 and pHC. The cells were cultured under normoxia and hypoxia and treated either with: A) DMSO or 75 µM resveratrol (Res) for the indicated time points; B) DMSO or different concentrations of Res for 24 h. PAI-1 protein was measured by Western blotting with an antibody against PAI-1 and blots were quantified by Fiji (NCBI). C) Representative Western blots with 75 µM resveratrol at 24 h for PAI-1, and blots were quantified by Fiji (NCBI). C) Representative Western blots with 75 µM resveratrol at 24 h for PAI-1, and blots were quantified by Fiji (NCBI). D) PAI-1 mRNA levels in HepG2 and pHC were measured by quantitative real-time PCR using SYBR green PCR mix and primers for PAI-1; β-Actin and Hprt (Hypoxanthine guanine phosphoribosyltransferase) were used as internal controls to normalize expression levels. The relative quantification of gene expression was determined using the ΔΔCt method; E, F) Cultured HepG2 cells (about 4×10⁵ cells/dish) or pHC (about 1×10⁶ cells/dish) were transfected for 5 h with 2.5 µg plasmid DNA containing 500 ng of a Renilla luciferase (RL) reporter plasmid (pRL-SV40, Promega) to control transfection efficiency and 2 µg of the human (E) or the rat (F) PAI-1 promoter Firefly luciferase construct. After 5 h, the medium was changed and the cells were cultured overnight. Then, 24 h before harvesting, resveratrol (75 µM) and in the controls DMSO were applied and cells were cultured under normoxia and hypoxia.
exerted by e.g. inflammatory mediators (21, 22) like TNF-α (23, 24), IL1β (25), or streptozotocin-induced type-1 diabetes (26) which is consistent with our present observation where resveratrol was more effective in non-cancerous cells stressed by hypoxia.

Unexpectedly, not much is known about resveratrol and regulation of PAI-1 in cancer cells. Only one study showed that resveratrol increased a PAI-1 promoter-Luc reporter construct in A549 cells in response to TGFβ (27). Our results are in line by showing that resveratrol enhances PAI-1 under normoxia and hypoxia in HepG2 cells. Thus, based on the limited data from the literature and our own study it appears that non-cancer cells and cancer cells produce PAI-1 in a different manner in response to resveratrol.

Overall, the observed opposite effects of resveratrol on PAI-1 provide important novel information about resveratrol in cancerous vs non-cancerous liver-derived cells under normoxia and hypoxia. Based on our own results and the available, still limited data from others, it is plausible to speculate that resveratrol is “beneficial” by suppressing the negative-prognosis marker PAI-1 in “healthy” cells under conditions of stress, e.g. hypoxia or inflammation. In contrast, in cancer cells resveratrol would induce PAI-1 and under a stress like hypoxia, this would lead to an additive up-regulation of PAI-1 with severe consequences for several processes regulated by PAI-1.

Altogether, our findings should alert the community and since resveratrol is currently available over-the-counter in Europe and USA as a dietary supplement, pose the question whether a general resveratrol supplementation is “beneficial” without knowing the physiological condition or health status of the individual to be treated. Moreover, the consequence of such resveratrol-dependent cell-type specific regulation may not only be important for PAI-1-regulation but also for a number of other clinically relevant proteins and disease markers.

Conflicts of interest
None declared.

References
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