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Resveratrol amplifies profibrogenic effects of free fatty acids on human hepatic stellate cells

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Abstract

Aim—To ascertain whether resveratrol affects the expression of free fatty acids (FFA)-induced profibrogenic genes, death receptors, and/or apoptosis-related molecules in human hepatic stellate cells, using the LX-2 cell line.

Methods—Cells were cultured in the presence of FFAs (2:1 oleate : palmitate) and subsequently treated with resveratrol. Gene expression rates were determined by quantitative realtime PCR. The 50% lethal dose (LD₅₀) of resveratrol in the presence of FFAs was assessed with the MTT viability test.

Results—Compared to vehicle controls, incubation of LX-2 cells with 0.5 mM FFAs induced profibrogenic genes (α -SMA $\times$ 2.9; TGF- β 1 $\times$ 1.6; TIMP-1 $\times$ 1.4), death receptors (CD95/Fas $\times$ 3.8; TNFR-1 $\times$ 1.4), and anti-apoptotic molecules (Bcl-2 $\times$ 2.3; Mcl-1 $\times$ 1.3). Subsequent addition of 15 μ M resveratrol (LD₅₀ = 23.2 μ M) significantly ($P < 0.05$) upregulated further these genes (α -SMA $\times$ 6.5; TGF- β 1 $\times$ 1.9; TIMP-1 $\times$ 2.2; CD95/Fas $\times$ 13.1, TNFR-1 $\times$ 2.1; Bcl-2 $\times$ 3.6; Mcl-1 $\times$ 1.9). Importantly, this effect was only observed in the presence of FFAs.

Conclusion—Resveratrol amplifies the profibrogenic activation of human hepatic LX-2 stellate cells. This finding raises the possibility that in obese patients with elevated FFAs resveratrol could provoke hepatic fibrogenesis. *In-vivo* studies are necessary to further validate this conclusion.

Keywords

resveratrol; free fatty acids; profibrogenic activation

Introduction

In 1979, ST Leger and colleagues found an inverse relationship between coronary heart disease and wine consumption;¹ this observation is now known as the “French Paradox”.² In the years following, further investigations revealing a potential benefit of components found in red wine (rather than in other alcoholic beverages) led to the “red wine hypothesis”.^{3,4} The beneficial effects of red wine were attributed to certain phenols that had been earlier identified by Frankel *et al.* as its dominant antioxidants.^{5,6} Since then, several effects could be associated with polyphenol intake, such as cardioprotection,⁷ the prevention of atherosclerosis⁷ and the promotion of NO release from vascular endothelial cells.⁸

More recently, resveratrol has been implicated as the most important polyphenol responsible for the beneficial effects of moderate red wine consumption. This non-flavonoid polyphenol exerts anti-oxidative,⁹ anti-neoplastic¹⁰ and anti-inflammatory properties.¹¹ It was further shown to have anti-fibrogenic activity by preventing TGF- β induced cardiac myofibroblast differentiation.¹² Also, resveratrol significantly reduced mortality, transaminase concentrations, and liver lesions in alcohol-treated mice,¹³ improved health and survival in obese mice,¹⁴ and was also suggested to act beneficially in chronic liver diseases.¹⁵ Resveratrol was further found to inhibit the proliferation of activated primary rat hepatic stellate cells,¹⁶ as well as of the activated murine hepatic stellate cell line, GRX;¹⁷ these findings suggested that resveratrol could therapeutically intervene with liver injury,¹⁶ and that polyphenol-rich foods may serve as an adjuvant treatment in chronic liver diseases.¹⁷

In contrast – and as prominent features of the non-alcoholic fatty liver diseases (NAFLD) – free fatty acids (FFAs) promote the process of liver fibrogenesis as they induce hepatocyte apoptosis¹⁸ and act profibrogenically.^{19,20} At least in part, these effects can be attributed to the increased expression of genes encoding for profibrogenic proteins, death receptors, and apoptosis-related molecules.¹⁸ Cumulatively, it thus seems possible that resveratrol might counteract certain detrimental consequences in liver that result from pathologically increased FFA levels. However, no data is available on the effect of resveratrol on fibrogenic cells in the presence of pathologic FFA concentrations.

In this study, we have explored the effects of resveratrol on the hepatic stellate cell, since this is the primary cell type implicated in fibrogenesis.^{21,22} Moreover, these cells have many more intrahepatic and systemic roles than previously assumed: they coordinate and integrate hepatic progenitor cell amplification and differentiation; they serve as a highly plastic functional nexus in a complex and tightly regulated sinusoidal milieu; and they function in a variety of metabolic and immunologic roles that contribute to overall homeostasis.²³

Given the emerging role of FFAs in fibrosis, and the suggestion that resveratrol may have therapeutic value, we have investigated the combined effects of FFAs and resveratrol on hepatic stellate cells. Specifically, should resveratrol be able to neutralize, or reduce, FFA-mediated detrimental effects in this crucial cell type, its benefit might even reach beyond protection from liver fibrogenesis. To our surprise, however, resveratrol appears to amplify the fibrogenic effects of FFAs.

Materials and Methods

LX-2 cells

LX-2 cells were cultured²⁴ at 2.5×10^6 /mL at total volumes of 14 mL in 75 cm² culture flasks in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 2 mM L-glutamine (PAA Laboratories, Pasching, Austria). Cell cultures were maintained in a 5% CO₂ atmosphere at 37°C in a steam-saturated incubator.

Incubation with FFAs and/or resveratrol

We employed freshly prepared solutions of FFAs, that is 2:1 oleate : palmitate (Sigma–Aldrich, Seelze, Germany) or of *trans*-resveratrol (i.e. (E)-5-(2-[4-hydroxyphenyl]ethenyl)-1,3-benzendiol) (Sigma–Aldrich) in phosphate-buffered saline without Ca²⁺ and Mg²⁺ (PBS) plus 1% fat-free albumin (Fraction V; Roche, Mannheim, Germany). Where indicated, LX-2 cells were cultured for 48 h in the presence of 0.5 mM FFAs and/or treated for an additional 24 h with or without resveratrol at 5, 10 or 15 μ M, respectively.

LD₅₀ of resveratrol

Based on Sagawa *et al.*,²⁵ we employed the MTT viability assay²⁶ to determine the 50% lethal dose (LD₅₀) of resveratrol in FFA-supplemented LX-2 cells. To this end, resveratrol was employed at concentrations of 0, 5, 10, 15, 20, 25, and 30 μ M under the general culture conditions specified above and in the presence of 0.5 mM FFAs. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, which is converted into its H₂O-insoluble derivative, 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan] (Sigma–Aldrich), was dissolved at 5 mg/mL in phosphate buffered saline (PBS) and employed without interim storage. At 24 h or 48 h after onset of culture, cells were centrifuged at 260 g, medium was removed, and 200 μ L of MTT solution was added per well. Cells were then incubated for an additional 26 h. After centrifugation at 260 g, removal of the MTT solution, and washing with PBS, MTT solubilization solution (10% Triton X-100 plus 0.01 N HCl in anhydrous isopropanol) was added to each flask. The viability rate of LX-2 cells was determined photometrically at an absorbance/optical density of 570 nm on a SpectraMax 250 microtiter plate reader (Molecular Devices, Sunnyvale, CA, USA).

Gene expression quantification

The mRNA levels of profibrogenic genes (α -SMA; TGF- β 1; TIMP-1), of death receptor genes (CD95/Fas; TNFR-1), and of genes encoding for anti-apoptotic molecules (Bcl-2; Mcl-1) were determined by quantitative realtime reverse-transcriptase PCR (qRT-PCR) using hypoxanthine phosphoribosyltransferase 1 (HPRT1) as a housekeeping gene (for primers, see Tab. 1). QRT-PCRs of the respective cDNAs were performed on an iQ thermal iCycler with real-time detection system software 3.0a and Genex software (BioRad, Hercules, CA, USA) in 30- μ L reactions containing 15 μ L QuantitTect Sybr Green master mix (Qiagen), 5 μ L cDNA, 1 μ L forward primer, 1 μ L reverse primer (at 10 pmol/ μ L, each) and 8 μ L *Aq. dest.* Amplifications were performed for 15 min at 95°C, followed by 40 cycles of 30 s at 95°C, 30 s at 55°C, and 30 s at 72°C. Melting-curve data were collected from 95°C to 55°C, at -0.5° C steps for 10 s, each. Relative gene expressions were calculated from the threshold cycles in relation to housekeeping gene or to untreated controls, respectively.

Detection of apoptosis

After 72 h of incubation with or without mixed FFAs and with or without resveratrol, LX-2 cultures were added 100 ng/mL of the CD95/Fas-stimulating mouse anti-human CD95/Fas-specific monoclonal antibody (clone CH11; Upstate Biochemicals, Lake Placid, NY, USA) for 4 h. CH11-negative controls revealed the unstimulated apoptosis rate in treated versus untreated cells. At the end of culture, cells were incubated with lysis buffer (R&D Systems) to generate cell lysates that were stored at -20° C until the assessment of apoptosis by M30 ELISA. The apoptosis marker, M30, was measured in LX-2 cells using the M30-Apoptosense ELISA kit (Peviva, Bromma, Sweden). The M30 neoepitope is only exposed upon cleavage of cytokeratin-18 (CK18) by activated caspase-3 at aspartate 396 in the process of apoptosis.²⁷ The M30 ELISA was chosen as a result of an initial comparison between the M30 and TUNEL assays, which revealed a superior specificity of M30 data.

Statistics

Descriptive statistics were performed for all variables; these include means and standard deviations or medians. Among experimental cell culture conditions, differences between FAT concentrations, death receptor expression rates and M30 neoepitope concentrations were evaluated statistically by one-way ANOVA, repeated-measures ANOVA or paired *t*-test. A *P*-value of ≤ 0.05 was considered as statistically significant. Analyses were performed with SPSS, version 15.0.1 (SPSS, Chicago, IL, USA).

Results

FFA uptake by LX-2 cells

Uptake of FFAs by LX-2 hepatic stellate cells after treatment with 0.5 mM of FFAs for 24 h was verified by fluorescence microscopy after Nile Red staining for lipids and blue nuclear counterstaining (Fig. 1). Negative control cells also revealed intracellular lipids – as, for example, obtained from the fetal bovine serum source – but lipid staining was much more intense in cells that had received additional FFAs.

Gene induction by FFAs in LX-2 cells

Relative to FFA-free controls, incubation of LX-2 cells with 0.5 mM FFAs resulted in an upregulation of the mRNAs of activation- and fibrosis-related genes (i.e. α -SMA: $\times 2.9$; TGF- β 1: $\times 1.8$; and TIMP-1: $\times 1.4$) by this stellate cell line (Fig. 2, left panel). The presence of additional FFAs also upregulated the expression of death receptor genes (i.e. CD95/Fas: $\times 3.8$; and TNFR-1: $\times 1.1$) (Fig. 3, left panel), as well as the expression of anti-apoptotic proteins (Bcl-2: $\times 2.3$; and Mcl-1: $\times 1.3$) (Fig. 3, left panel). Of all mRNAs investigated, CD95/Fas was upregulated most in the presence of 0.5 mM FFAs when compared to the negative controls. This is in line with our results in the HepG2 hepatoblastoma cell line representing hepatocytes.

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Gene induction by resveratrol in LX-2 cells

Under FFA-supplemented conditions mimicking steatosis *in vitro*, resveratrol was first employed at concentrations of up to 30 μ M for determining the 50% lethal-dose level. In the presence of 0.5 mM FFAs, the LD₅₀ of resveratrol in human LX-2 hepatic stellate cells was thus calculated as 23.2 μ M.

Next, resveratrol was employed at concentrations of 0 (negative control), 5, 10, or 15 μ M, respectively. The most distinct effects were measured at 15 μ M for all resveratrol-treated conditions tested (see below). Importantly, this finding corresponds to results by Lancon *et al.* and Baur *et al.* who showed that correlating concentrations proved beneficial in mice when applied *in vivo*.^{14,29} We thus selectively depict results obtained with 15 μ M of resveratrol. At lower concentrations, the parameters' expression rates were less pronounced than at 15 μ M, but they did not differ qualitatively (not shown).

As shown in Figures 2 and 3, resveratrol upregulated the expression of key mRNAs associated with activated, fibrogenic stellate cells. However, this effect was less marked than in the presence of FFAs. Specifically, among the activation- and fibrosis-related genes, only α -SMA and TIMP-1 were slightly upregulated (i.e. α -SMA: $\times 1.7$; and TIMP-1: $\times 1.4$) (Fig. 2, central panel); among the death receptor genes, only CD95/Fas was upregulated by $\times 2.2$ (Fig. 3, central panel); and only one of the anti-apoptotic molecules, Bcl-2, was found increased by $\times 1.6$ (Fig. 3, central panel). Therefore, and despite statistical significance in the cases of α -SMA and CD95/Fas (see Figs 2,3, central panels), resveratrol alone induced gene expression much less than FFAs. Nevertheless, and similar to the findings in the presence of FFAs only, resveratrol again had the greatest influence on the expression of CD95/Fas mRNA; this supports the view that CD95/Fas is highly sensitively upregulated by a broad spectrum of chemically diverse compounds.

Combined effect of FFAs and resveratrol on LX-2-cell gene expression

Importantly, pre-incubation of LX-2 cells with FFAs before adding resveratrol mimics the conditions encountered by hepatic stellate cells in obese patients who consume red wine for pleasure and/or because of its presumptive health benefits on a background of consistently elevated serum FFA levels. Our *in-vitro* results indicate that such a concurrent presence of

FFAs and resveratrol may enhance the degree of activation in hepatic stellate cells and, hence, their pro-fibrotic activity. When human LX-2 stellate cells had already been incubated for 48 h with 0.5 mM of FFAs, further addition of 15 μ M resveratrol significantly ($P < 0.05$) upregulated the expression of all of the genes investigated. Of note, the expression rates of these genes not only increased in comparison to the negative control values, but also relative to the expression rates determined in cells that received FFAs only. Specifically, mRNA expression of activation- and fibrosis-related genes were upregulated (i.e. α -SMA: $\times 6.5$; TGF- β 1: $\times 1.9$; and TIMP-1: $\times 2.2$; Fig. 2, right panel), as were those of death receptor genes (i.e. CD95/Fas: $\times 13.1$; and TNFR-1: $\times 2.1$; Fig. 3, right panel), as well as the mRNA expressions of genes encoding for anti-apoptotic molecules (i.e. Bcl-2: $\times 3.6$; and Mcl-1: $\times 1.9$; Fig. 3, right panel).

Susceptibility to apoptosis

As depicted in Figure 4, the presence of FFAs or of resveratrol alone, or the combined presence of FFAs and resveratrol significantly reduced ($P < 0.05$) the hepatic stellate cells' susceptibility to apoptosis. This finding applied both to the spontaneous rates of apoptosis under either of these conditions, as well as to the provoked induction of apoptosis by the CD95/Fas-agonistic monoclonal antibody, CH11. Even more importantly, under both principal conditions – that is unprovoked and antibody-stimulated apoptosis – the degrees of apoptosis determined for LX-2 cells treated with both FFAs and resveratrol were significantly different ($P < 0.05$) than cells incubated in the presence of FFAs only.

Discussion

Hepatic stellate cells are of central importance for the induction and propagation of liver fibrosis, and they provide an increasingly recognized plethora of functions for the liver and the entire organism.²³ It thus appeared rewarding to investigate the combined effect of resveratrol and pathologic FFA concentrations on a human stellate cell line. Indeed, this study for the first time investigated the effect of resveratrol on human hepatic stellate cells that were adjusted to mimic the state in morbidly obese individuals by providing FFA-enriched conditions.

Our working hypothesis suggested that resveratrol might counteract the undesirable FFA-mediated activation of this crucial cell type. To verify this assumption could have important implications for the (adjuvant) treatment of patients with chronic liver diseases as, for example, had been suggested by Souza *et al.*¹⁷ Yet, our results revealed a different story. Indeed, FFAs activated human LX-2 stellate cells, which is consistent with results by Lu *et al.* in primary rat hepatic stellate cells.²⁰ However, the subsequent addition of resveratrol further exacerbated the FFAs' detrimental effects. This red-wine component not only amplified the expression of some select genes, but even of all three groups of genes investigated. The strongest amplification rates were found for the death receptor, CD95/Fas, as well as for the profibrogenic protein α -SMA; however, all other transcripts, including the anti-apoptotic mediators Bcl-2 and Mcl-1 were also super-induced in the parallel presence of FFAs and resveratrol. As a result of the induction of anti-apoptotic key factors, the apoptosis rate of LX-2 cells, as measured by an M30 immunoassay, was found decreased in cells treated with FFAs only, but even more so in cells treated with both FFAs and resveratrol. Still, it appears unlikely that slight increases of only Bcl-2 and Mcl-1 may have so efficiently counter-regulated the sharp upregulation of CD95/Fas. Yet this finding strongly suggests that other pro-apoptotic mediators that were not determined here are also upregulated. As the results argue for themselves, further experimental insight will thus likely reveal that, in fact, various anti-apoptotic factors in concert induce in FFA-laden hepatic stellate cells exposed to resveratrol a reduced susceptibility to die from apoptosis.

Interestingly, both measured on day 3 of culture, Kawada *et al.* had found suppression of α -SMA expression in the presence of 100 μ M resveratrol¹⁶ while we arrived at exactly the opposite result at 15 μ M resveratrol in the presence of FFAs. However, these results are not easily compared. Basically, it is known that stellate cells obtained from different species may differ in distinct properties as, for example, shown by Zhou *et al.*³⁰ This indicates that the putatively antagonistic effect of resveratrol may, at least in part, be due to the fact that the compared studies employed rat versus human hepatic stellate cells. Still, as the concentrations employed differ by almost one order of magnitude, it were improper to directly compare their effect. However, our study specifically aimed at identifying effects of resveratrol on an FFA-laden human stellate cell line as a correlate of such cells in obese individuals. Our key finding is thus that resveratrol exacerbates the FFAs' profibrogenic effect. Nevertheless, we still subscribe to the conclusion by Kawada and colleagues that resveratrol might interfere with the stellate cells' signal transduction,¹⁶ albeit in a dissimilar manner in cells obtained from different species, and obviously depending on the nature of the (pre-)activating stimulus, such as pathologically elevated FFA levels.

A number of studies demonstrated positive effects of resveratrol as well as, likely, other polyphenols on various non-liver diseases;^{2,3,5–10,12} these findings suggested that the consumption or application of resveratrol – whether in the form of red wine or as an isolated substance – may also act beneficially in obesity, upon ethanol abuse, as well as in liver disease, which was supported by results in mice.^{11,13} However, our findings in human stellate cells contradict both such assumptions, as well as the results obtained in rodents.

The role of FFAs in chronic liver diseases, and most prominently in NAFLD, has been extensively characterized as, for example, in a lipidomic analysis by Puri *et al.*³¹ Insight from such studies revealed that the detrimental activity of FFAs is most likely mediated *via* their induction of pro-apoptotic features in hepatocytes.^{18,19} Moreover, Kupffer cells that are activated in liver injury produce chemokines and over-express death ligands (e.g. FasL, TNF- α , and TRAIL) which induce apoptosis in hepatocytes.^{32,33} When viewed from this perspective, one might reason that the suppression of apoptosis, as was most strikingly achieved in LX-2 cells in the presence of both FFAs and resveratrol, might be advantageous for patients with fibrotic liver diseases. Yet this response appears restricted to hepatocytes¹⁸ while the opposite applies to hepatic stellate cells. As shown following partial hepatectomy, death receptors and their ligands can actually promote liver regeneration.³⁴ However, hepatic stellate cells produce collagen in chronic liver injury so as to replace sites of hepatocyte death and liver injury. The activation of these cells, by promoting fibrogenesis, thus is the dominant event in liver fibrosis. In this context, induction of hepatic stellate-cell apoptosis is a desirable therapeutic strategy that promises to indirectly downregulate collagen production by these cells and thus to counteract liver fibrogenesis.³⁵ Thus, the combined action of FFAs and resveratrol may exacerbate fibrogenesis. In addition, under these conditions the expression of all of the genes investigated – with some of which known to actively participate in the progression of liver fibrosis – were upregulated.

Notwithstanding these considerations, the actual *in-vivo* setting is probably more complex and may require the integration of additional factors, such as, but not limited to, the following:

- i. Besides their combined action on hepatic stellate cells as the pathogenetically predominant cell class in liver fibrosis, one may expect further potentially powerful effects of FFAs and resveratrol on other liver-composing cell types. However, apart from the study by Baur *et al.* that demonstrated prevention by resveratrol from the development of fatty liver in mice,¹⁴ we are not aware of studies in humans or animals addressing combined FFA/resveratrol effects on hepatocytes or other liver cell species. In the absence of empirical data it were inadequate to speculate on such effects because of the unpredictable nature of potential mutual interactions between the

different cell types *in vivo*. Such effects and interactions must be addressed empirically.

- ii. The detrimental “resveratrol effect” first described herein became obvious only in the presence of FFAs. This interesting discovery might hint towards a direct lipophilic interaction between these molecules. Such interaction would imply the formation of molecular aggregates with altered binding kinetics to, for example, cellular surface receptors and/or fatty acid transport proteins. This may entail modified signal transduction as had already been suggested by Kawada and colleagues.¹⁶
- iii. Irrespective of whether such aggregates are formed, one might also speculate on an additive cytotoxic effect by FFAs and resveratrol, but we never observed such an outcome. Yet it would not appear surprising to discover further unknown interactions of polyphenols with other molecules that may provoke detrimental consequences, such as the induction of apoptosis in select liver-composing cell species with a proven key role in the pathogenesis of liver fibrosis.^{19,28,32,33} Indeed, such an effect, if verified, would diametrically oppose the suggested protective potential of resveratrol.
- iv. Another important aspect may be the role of biotransformation. For example, Yu *et al.* found that – in humans, rats and mice alike – the most abundant metabolites formed to excrete free resveratrol are *trans*-resveratrol-3-*O*-glucuronide and *trans*-resveratrol-3-sulfate.³⁶ In contrast, there is obviously as yet no data available on the biotransformation of presumptive FFA-resveratrol aggregates.

Based on both this data and considerations, the recommendation of moderate consumption of red wine to obese individuals should be reconsidered until *in vivo* models can be used to further clarify this issue. The detrimental “resveratrol effect” is only apparent in the presence of FFAs, which could hint towards a direct lipophilic interaction between these molecules, which requires further analysis.

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Figure 1.

Incubation human LX-2 hepatic stellate cells in the absence or presence of free fatty acids (FFAs; 0.5 mM) with or without the principal concentration of resveratrol (Res.; 15 μM). Untreated negative controls (upper left-hand side) as well as treated cells and are shown as doublets, each, i.e. by phase-contrast microscopy (top), and by fluorescence microscopy showing Nile-Red lipid staining and blue nuclear counterstaining with 4'-6-diamidino-2-phenylindole (DAPI) (bottom). The morphology and viability of cells treated with 15 μM of resveratrol did not differ from cells maintained without this polyphenol. All photographs are representative of multiples of cultures kept under the respective condition (bar = 20 μm).

Figure 2.

Expression of activation- and fibrosis-related genes in LX-2 cells. The expression of mRNAs in FFA-treated (left), resveratrol-treated (center), and co-treated cells was determined by quantitative real-time reverse-transcriptase polymerase chain reaction (qRT-PCR). Asterisks indicating statistical significance refer to the faintly shaded negative controls kept without FFAs or resveratrol. Results were reproducible in all of the cases. See main text for detailed information and interpretation.

Figure 3.

Expression of death receptor genes (CD95/Fas, TNFR-1) and of genes encoding for anti-apoptotic receptors (Bcl-2, Mcl-1) in LX-2 cells. Results were reproducible in all of the cases. For further specifications, see caption to Fig. 2. Detailed information and interpretation is given in the main text.

Figure 4.

Determination of unstimulated and stimulated apoptosis in LX-2 hepatic stellate cells. Apoptosis was provoked *via* the CD95/Fas-agonistic monoclonal antibody, CH11. Asterisks indicating statistical significance refer to connected columns. Results were reproducible in all of the cases. See main text for detailed information and interpretation.