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Up-regulation of endothelial nitric oxide synthase (*eNOS*), silent mating type information regulation 2 homologue 1 (*SIRT1*) and autophagy-related genes by repeated treatments with resveratrol in human umbilical vein endothelial cells

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Abstract

Resveratrol, a polyphenolic phytoalexin found in red wine and various plants, has been reported to up-regulate the expression of endothelial NO synthase (eNOS) in human umbilical vein endothelial cells (HUVEC). However, this effect was neither long term in nature nor physiologically relevant at the concentration of resveratrol studied. In the present study, we investigated the effects of repeated treatments with a lower concentration of resveratrol on the expression of genes in HUVEC. The expression levels of eNOS and silent mating type information regulation 2 homologue 1 (SIRT1) were up-regulated in HUVEC by repeated treatments with 1 μM-resveratrol for 6 d, but not with fenofibrate. Moreover, resveratrol treatment increased the expression of autophagy-regulated genes such as γ-aminobutyric acid A receptor-associated protein (*GABARAP*), microtubule-associated protein 1 light chain 3B (*LC3B*) and autophagy-related protein 3 (*ATG3*), the radical scavenger activity-related metallothionein-1X (*MT1X*) gene and the anti-inflammatory activity-related annexin A2 (*ANXA*) gene. In addition, resveratrol treatment down-regulated the expression of the cell-cycle checkpoint control RAD9 homologue B (*RAD9B*) gene. These results indicate the beneficial effects of resveratrol on the cardiovascular system.

Key words: Resveratrol: Endothelial nitric oxide synthase: Silent mating type information regulation 2 homologue 1: Autophagy

The prevention of lifestyle-related diseases such as CVD, diabetes and stroke has attracted worldwide interest. Current treatment regimens for lifestyle-related diseases have shifted their focus onto the functionality of natural chemicals present in foods and drinks. One example is the association between the long-term consumption of red wine and reduced risk for CVD. In this context, resveratrol, a phytoalexin and antioxidant polyphenol present in red wine and various plants, has emerged as one of the most attractive and extensively studied compounds[1,2].

We have previously demonstrated that resveratrol suppresses the expression of cyclo-oxygenase (COX)-2, the rate-limiting enzyme in PG biosynthesis[3], particularly in 184B5/HER(184B5/human EGFR-related-2)-transformed mammary epithelial cells[4]. Additionally, we have shown that resveratrol activates PPARα, PPARβδ and PPARγ[5,6], which are members of the nuclear receptor family of ligand-dependent transcription factors[7,8]; that it protects the brain against ischaemic stroke in mice through a PPARα-dependent mechanism demonstrated in cell-based reporter assays[5] and that it acts via a negative feedback loop mediated through PPARγ to inhibit the expression of COX-2, particularly in macrophages[9]. Based on these data, we have focused our attention on PPAR as potential molecular targets of resveratrol in the prevention of lifestyle-related diseases, the molecular mechanisms of which remain to be determined.

Previous studies have investigated the effects of resveratrol on the activation of silent mating type information regulation 2 homologue 1 (SIRT1), a NAD^+-dependent protein

Abbreviations: BAEC, bovine arterial endothelial cells; COX, cyclo-oxygenase; eNOS, endothelial nitric oxide synthase; HUVEC, human umbilical vein endothelial cells; RAD9B, RAD9 homologue B; SIRT1, silent mating type information regulation 2 homologue 1.

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deacetylase[10–12], although a direct activation was not observed[13,14]. Resveratrol has been reported to enhance the expression of endothelial NO synthase (eNOS)[15], which may be important for elucidating the effects of resveratrol on the cardiovascular system. However, these observations were based on human umbilical vein endothelial cells (HUVEC) treated with 33 μM-resveratrol for 24 h, conditions which are neither long term in nature nor physiologically relevant, and a time (24–72 h)- and concentration-dependent up-regulation of the expression of eNOS (2·8-fold in 100 μM-resveratrol) was observed not in HUVEC but in the EA.hy926 cell line, which is a hybrid of the HUVEC and human lung carcinoma A549 cells[15].

In the present study, we investigated the effects of resveratrol on the expression of eNOS using a physiological concentration (1 μM) during repeated treatments for 6 d. We demonstrated that the expression of eNOS and SIRT1 is up-regulated by resveratrol. We screened the differentially regulated genes in HUVEC on resveratrol treatment using microarray analysis and confirmed the candidate genes using quantitative RT-PCR analysis. We demonstrated that resveratrol increases the expression of autophagy-related genes, γ-aminobutyric acid A receptor-associated protein (*GABARAP*), microtubule-associated protein 1 light chain 3B (*LC3B*) and autophagy-related protein 3 (*ATG3*), and the radical scavenger activity-related metallothionein-1X (*MT1X*) gene. Additionally, we demonstrated that the expression of the cell-cycle checkpoint control RAD9 homologue B (*RAD9B*) gene is down-regulated by resveratrol. These data help in the identification of molecular mechanisms that may contribute to the beneficial effects of resveratrol on the cardiovascular system.

Experimental methods

Cell culture

HUVEC were obtained from the Japanese Collection of Research Bioresources and were grown in an endothelial cell growth medium (Cell Applications, Inc.). The cells were cultured for 6 d in the presence or absence of 1 μM-resveratrol or 1 μM-fenofibrate, a synthetic PPARα agonist, with the media being changed at 0, 2 and 4 d.

RNA extraction and analysis

Total RNA was isolated from the HUVEC using the acid guanidinium thiocyanate procedure and was analysed for gene expression via real-time quantitative RT-PCR (Mx3005; Stratagene) as described previously[16]. The primer pairs for the genes used in the present study and the cycling conditions are given in Table S1 (available online). The expression level of each mRNA was normalised to that of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA, which has been used for our previous study in HUVEC[5,17]and bovine arterial endothelial cells (BAEC)[9,16].

DNA microarray analysis

HUVEC were cultured for 6 d in the presence or absence of 1 μM-resveratrol. DNA microarray analysis was performed as described previously[18]. Briefly, poly(A)$^+$ RNA was prepared from resveratrol-treated and untreated HUVEC using a QuickPrep micro mRNA purification kit (GE Healthcare) and was used to prepare complementary RNA samples as described in the GeneChip Expression Analysis Technical Manual (Affymetrix). Double-stranded complementary DNA was synthesised from 0·6 μg of poly(A)$^+$ RNA using a Message-AMP™ II aRNA amplification kit (PE Applied Biosystems) with T7-$(dT)_{24}$ primer (GE Healthcare). The product served as a template for the synthesis of biotin-labelled complementary RNA via *in vitro* transcription using a GeneChip IVT labelling kit (Affymetrix), and the amplified complementary RNA was fragmented by heat treatment in the presence of potassium acetate and magnesium acetate. The complementary RNA sample (10 μg) was then applied to a GeneChip Human Genome U133 Plus 2.0 Array (Affymetrix), and hybridisation, washing, staining and scanning were performed according to the GeneChip Manual. The data for each probe set were calculated from the scanned array image using the GeneChip Analysis Suite (Affymetrix) and GeneSpring (Agilent Technologies). The procedure of analysis was as follows: for background subtraction, the median value of the negative control genes was subtracted from the raw values for each gene, and the resulting values were divided by the value of the 50th percentile of each chip to normalise the various signal intensities among the arrays. Finally, the ratios (resveratrol-treated:untreated and untreated:resveratrol-treated) were calculated from the average of duplicate normalised signal intensities. A change in expression of 1·5-fold was used as a threshold for comparison.

Statistical analysis

All results are expressed as the means and standard deviations. Comparisons between the groups were made using one-way ANOVA with *post hoc* Bonferroni multiple-comparison test or the unpaired *t* test. A P value $<0·05$ was considered to indicate statistical significance.

Results and discussion

Repeated treatments with resveratrol up-regulate the expression of endothelial nitric oxide synthase

HUVEC were cultured for 6 d in the presence or absence of 1 μM-resveratrol and examined for the expression of eNOS by quantitative RT-PCR (Fig. 1(a)). The levels of *eNOS* mRNA were up-regulated 1·5-fold. Previous studies have demonstrated approximately 1·4-fold up-regulation of *eNOS* mRNA levels in HUVEC following treatment with 33 μM-resveratrol, but not with 1 μM, for 24 h[15], indicating that long-term repeated treatments with 1 μM-resveratrol for 6 d, as done in the present study, lead to the up-regulation of *eNOS* mRNA levels. Our results are in agreement with the report that daily treatment with resveratrol significantly increases the production of both eNOS protein and NO in HUVEC[19]. Thus, induction of the expression of eNOS by repeated treatments with a physiological concentration of

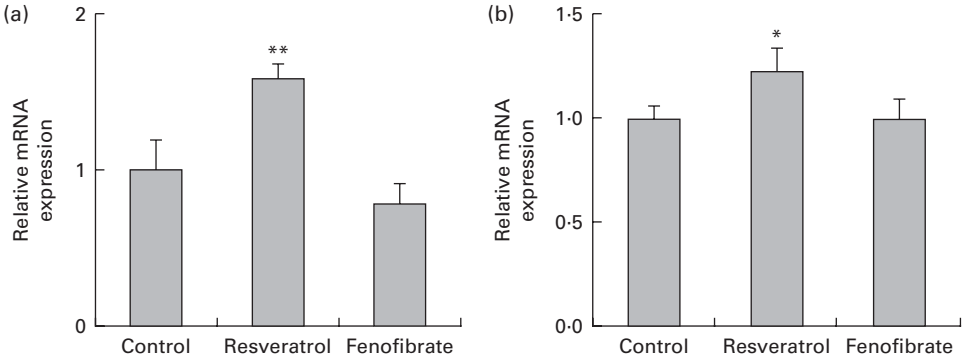

Fig. 1. Up-regulation of the expression of endothelial nitric oxide synthase (*eNOS*) and silent mating type information regulation 2 homologue 1 (*SIRT1*) mRNA and protein in human umbilical vein endothelial cells (HUVEC) following repeated treatments with 1 μM-resveratrol for 6 d. HUVEC were cultured for 6 d in the presence or absence of 1 μM-resveratrol or 1 μM-fenofibrate. Isolated RNA from the HUVEC was used for quantitative RT-PCR analysis. The mRNA levels of (a) *eNOS* and (b) *SIRT1* were normalised to that of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. The normalised levels of *eNOS* and *SIRT1* mRNA in the absence of resveratrol are expressed as 1, respectively. Values are means of three separate dishes, with standard deviations represented by vertical bars. Mean values were significantly different from those of the control and fenofibrate treatments: * $P<0·05$ and ** $P<0·01$ (one-way ANOVA with *post hoc* Bonferroni multiple-comparison test). Similar results were obtained by two additional experiments.

resveratrol can account, in part, for the cardiovascular benefits of the long-term consumption of red wine.

Repeated treatments with resveratrol up-regulate the expression of silent mating type information regulation 2 homologue 1

We examined the expression levels of *SIRT1* mRNA (Fig. 1(b)) in HUVEC treated with 1 μM-resveratrol for 6 d. The levels of *SIRT1* mRNA were up-regulated by 1·2-fold. As previous studies have reported that resveratrol is not a direct activator of SIRT1[13,14], we sought to investigate the mechanism of the up-regulation of SIRT1 levels. We have previously demonstrated that resveratrol is a triple agonist for PPARα, PPARβ/δ and PPARγ *in vitro* [5,6] and that 4-week intake of resveratrol as well as the synthetic PPARα agonist fenofibrate up-regulates the hepatic expression of SIRT1 in wild-type, but not in *PPARα* knockout, mice (R Nakata *et al.*, unpublished results). Therefore, we examined the expression of SIRT1 in HUVEC treated with 1 μM-fenofibrate for 6 d and observed that *SIRT1* mRNA levels were unregulated by fenofibrate (Fig. 1(b)). Similarly, no effect on the expression of eNOS was observed following fenofibrate treatment (Fig. 1(a)). These discrepancies between the present study using HUVEC *in vitro* and the *PPARα* knockout mice study *in vivo* indicate the limitations of the present study. Cell culture of HUVEC is more difficult than that of BAEC since the former need several special supplements such as epidermal growth factor, basic fibroblast growth factor and vascular endothelial growth factor. Moreover, there are no interactions between HUVEC and other types of cells, which is common *in vivo*. On the other hand, fasting is reported to promote the expression of SIRT1 via the activation of PPARα in mice[20], whereas there is no report of a connection between the up-regulation of eNOS levels and the activation of PPARα. Therefore, we could

Table 1. Representative genes differentially expressed following repeated treatments with resveratrol

Gene	Fold change	GenBank no.
Up-regulated		
Human DNA sequence from clone RP4-581F12 on chromosome Xq21	× 3·6	AL031313
γ-Aminobutyric acid A receptor-associated protein (*GABARAP*)	× 2·4	NM_001307
Hypothetical protein LOC134466	× 2·3	AI242408
α-Tubulin-like	× 2·3	NM_145042
Human DNA sequence from clone RP5-1118D24 on chromosome 1p36.11–36·33	× 2·3	AL031276
Homo sapiens DNA-damage-regulated autophagy modulator 1 (*DRAM1*)	× 2·2	NM_018370
Annexin A2 (*ANXA2*) or lipocortin 2	× 2·1	M62895
Homo sapiens angiomotin-like 1 (*AMOTL1*)	× 2·1	NM_130847
Metallothionein-1X (*MT1X*)	× 1·8	NM_005952
Glutathione *S*-transferase pi 1 (*GSTP1*)	× 1·5	NM_000852
Down-regulated		
Myocardin-related transcription factor B (*MRTFB*)	× 0·26	AK093577
RAD9 homologue B (*RAD9B*)	× 0·29	AK058176
Clone IMAGE:4794726	× 0·36	BF513121
Hypothetical protein MGC34713	× 0·36	NM_173665
Small nuclear ribonucleoprotein polypeptide N	× 0·37	AU118874
Hypothetical gene supported by AK093253 (*LOC400579*)	× 0·38	BC033201
Cyclin-dependent kinase 6	× 0·59	AW192700
Homo sapiens ATG3 autophagy-related 3 homologue (*ATG3*)	× 0·59	NM_022488

not answer why the expression of eNOS and SIRT1 was induced without involving PPAR due to the limitations of the present study.

Repeated treatments with resveratrol up-regulate the expression of autophagy-, radical scavenger activity- and anti-inflammatory activity-related genes and down-regulate the expression of a cell-cycle checkpoint control gene

To examine the expression of other differentially regulated genes following resveratrol treatment, DNA microarray analysis was performed. Using cut-off values of 1·5-fold for induction and 0·67-fold for suppression, 256 up-regulated and 411 down-regulated genes were identified. Among these candidate genes (Table 1), the up-regulation of the autophagy-related *GABARAP* gene[21] was confirmed by real-time quantitative RT-PCR (Fig. 2(a)). The induction of autophagocytosis by 50 μM-resveratrol and the extension of the autophagy-mediated lifespan have been reported previously[22,23]. In this context, we found that the expression of the autophagy-related *LC3B* and *AGT3* genes was also up-regulated by treatment with 1 μM-resveratrol for 6 d (Fig. 2(b) and (c)), although these were not detected in the microarray analysis. Autophagy plays key roles in adaptation to stress such as starvation and normal development of the immune system as well as in a wide range of disease states. Therefore, the beneficial effects of resveratrol could partly be explained by the up-regulation of the expression of autophagy-related genes (Fig. 2). On the other hand, the beneficial effects of fenofibrate on retinal pigment epithelium have recently been reported to be partly due to the induction of autophagy[24]; however, we could not observe the induction of the expression of autophagy-related genes by fenofibrate under our assay condition (data not shown).

The up-regulation of the expression of the radical scavenger activity-related *MT1X* (Fig. 3(a)) and anti-inflammatory activity-related annexin A2 (*ANXA2*; Fig. 3(b)) genes and the down-regulation of the expression the *RAD9B* gene (Fig. 3(c)) were also observed using real-time quantitative RT-PCR analysis. ANXA2 is reported to be a regulator of cell surface plasmin generation[25], and there are no reports on the function of RAD9B, but its gene is a homologue of *RAD9*, an evolutionarily conserved gene with multiple functions for preserving genomic integrity[26], indicating that the beneficial effects of resveratrol may partly be due to the up-regulation of the expression of the antioxidant *MT1X* and anti-inflammatory *ANXA2* genes and the down-regulation of the expression of the *RAD9B* gene. However, there were several discrepancies between the DNA microarray (Table 1) and real-time quantitative RT-PCR results for the angiomotin-like 1 (*AMOTL1*; Fig. 3(d)), *LOC134466* (Fig. 3(e)), glutathione *S*-transferase pi 1 (*GSTP1*; Fig. 3(f)), myocardin-related transcription factor B (*MRTFB*; Fig. 3(g)) and cyclin-dependent kinase 6 (*CDK6*; Fig. 3(h)) genes. Similarly, induction of the expression of the *eNOS* and *SIRT1* genes by resveratrol was detected using real-time quantitative RT-PCR (Fig. 1) but not by the microarray analysis. These results show that the DNA

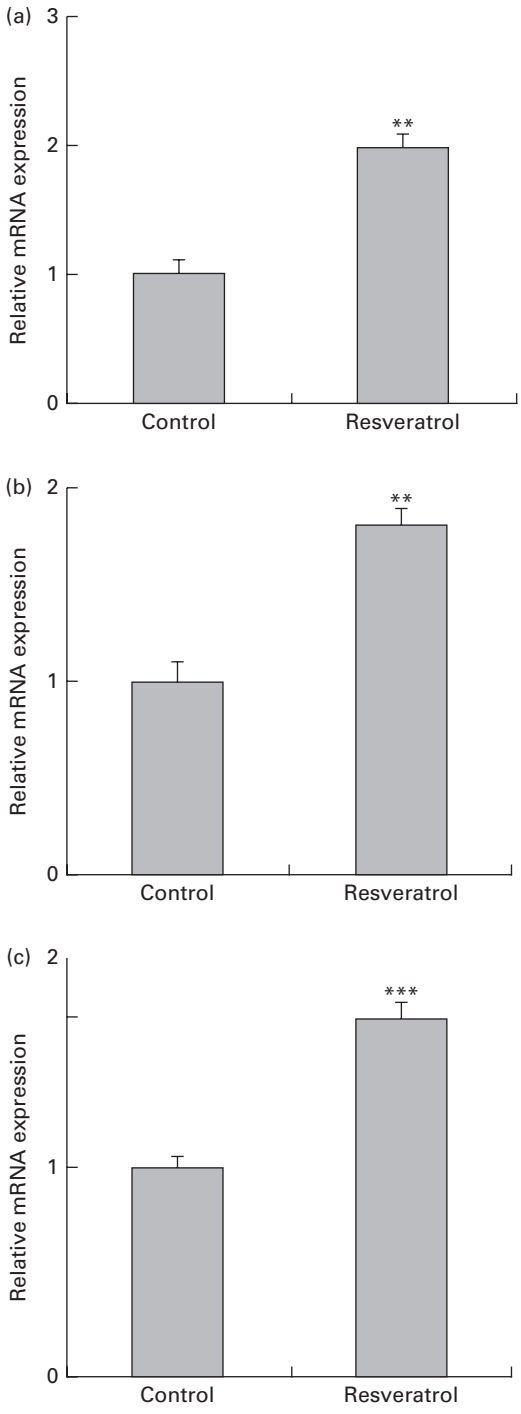

Fig. 2. Up-regulation of the expression of autophagy-related genes in human umbilical vein endothelial cells (HUVEC) following repeated treatments with 1 μM-resveratrol for 6 d. HUVEC were cultured in the presence or absence of 1 μM-resveratrol as described in Fig. 1, and isolated mRNA from the HUVEC was used for quantitative RT-PCR analysis. The level of each mRNA was normalised to that of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. The normalised levels of the control mRNA are expressed as 1. The relative mRNA expression levels of (a) γ-aminobutyric acid A receptor-associated protein (*GABARAP*), (b) microtubule-associated protein 1 light chain 3B (*LC3B*) and (c) autophagy-related protein 3 (*ATG3*) are shown. Values are means of three separate dishes, with standard deviations represented by vertical bars. Mean values were significantly different from those of control: ** $P<0·01$ and *** $P<0·001$ (unpaired *t* test). Similar results were obtained by two additional experiments.

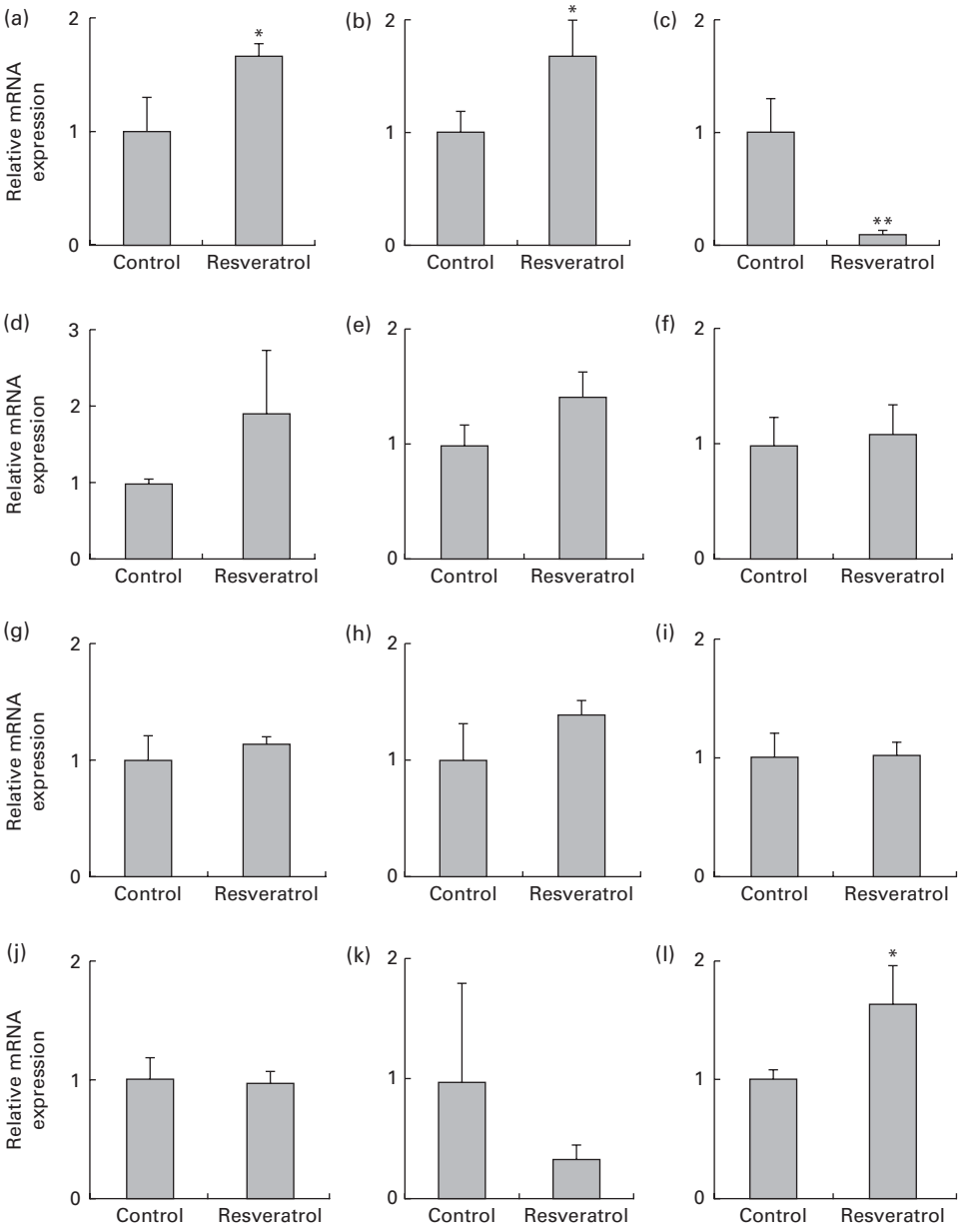

Fig. 3. Expression of the radical scavenger activity-, anti-inflammatory activity- and cell-cycle checkpoint control-related genes and cyclo-oxygenase 2 (*COX-2*), *PPARα*, *PPARβ/δ* and *PPARγ* genes in human umbilical vein endothelial cells (HUVEC) following repeated treatments with 1 μM-resveratrol for 6 d. The mRNA was isolated from the HUVEC for quantitative RT-PCR analysis following treatment with 1 μM-resveratrol for 6 d. The level of each mRNA was normalised to that of the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) mRNA. The relative mRNA expression levels of (a) metallothionein-1X (*MT1X*), (b) anti-inflammatory activity-related annexin A2 (*ANXA2*), (c) RAD9 homologue B (*RAD9B*), (d) angiomotin-like 1 (*AMOTL1*), (e) *LOC134466*, (f) glutathione *S*-transferase pi 1 (*GSTP1*), (g) myocardin-related transcription factor B (*MRTFB*), (h) cyclin-dependent kinase 6 (*CDK6*), (i) *COX-2*, (j) *PPARα*, (k) *PPARβ/δ* and (l) *PPARγ* are shown. Values are means of three separate dishes, with standard deviations represented by vertical bars. Mean values were significantly different from those of control: * $P<0{\cdot}05$ and ** $P<0{\cdot}01$ (unpaired *t* test). Similar results were obtained by two additional experiments.

microarray analysis is useful for the screening of candidate genes, but this should be confirmed by real-time quantitative RT-PCR.

Finally, we examined the expression of COX-2 and PPAR. The expression of COX-2 was unchanged following resveratrol treatment (Fig. 3(i)). This is consistent with our previous observations that resveratrol suppresses the expression of COX-2 in 184B5/HER-transformed mammary epithelial cells[4], but not in BAEC (H Inoue, unpublished results), and that 15-deoxy-$\Delta^{12,14}$ PGJ_2, a natural PPARγ agonist, suppresses the expression of COX-2 in macrophage-like U937 cells, but not in BAEC[9]. The expression levels of PPARα (Fig. 3(j)) and PPARβ/δ (Fig. 3(k)) were also unchanged following resveratrol treatment; however, the expression of PPARγ was up-regulated (Fig. 3(l)). Increased expression of PPARγ had not been detected previously in BAEC[9], indicating possible differences between these cell lines.

In summary, we demonstrated that the expression of eNOS and SIRT1 is up-regulated in HUVEC following repeated treatments with a physiological concentration (1 μM) of

resveratrol for 6 d. These results will account, in part, for the cardiovascular benefits of the long-term consumption of red wine. Moreover, genes related to autophagy, radical scavenger activity, anti-inflammatory activity and cell-cycle checkpoint control were shown to be differentially regulated in this condition. Modulating the expression of these genes may contribute to the observed beneficial effects of resveratrol on the cardiovascular system.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114513001670

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