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In Vivo, in Vitro, and in Silico Studies of Cu/Zn-Superoxide Dismutase Regulation by Molecules in Grape Seed Procyanidin Extract

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The potential beneficial effects of flavonoids on human health have aroused considerable interest and were initially attributed to their antioxidant activities. Recent studies have speculated that as well as their antioxidant role, flavonoids can act by modulating cell signaling pathways and/or gene expression. In this respect, we have used streptozotocin-induced diabetic rats as an oxidative stress model to study whether grape seed procyanidin extract (GSPE) regulates copper/zinc-superoxide dismutase (Cu/Zn-SOD), an enzyme that defends against oxidative stress. The results indicate that the expression profile of Cu/Zn-SOD in diabetic rats was similar to the profile in nondiabetic rats. Nevertheless, the administration of GSPE increased Cu/Zn-SOD activity in both diabetic and nondiabetic rats. Therefore, to evaluate whether this increase in activity was dose-dependent, we also studied the effect of GSPE on Cu/Zn-SOD expression by using an in vitro model (Fao cell line hepatocytes). The cells were exposed to GSPE doses between 0 and 150 mg/L for 24 h, and the results showed that enzyme activity was enhanced only with 15 mg/L of GSPE. Therefore, we decided to explore whether this increase in Cu/Zn-SOD activity was due to direct interaction between some of the molecules in GSPE and the enzyme (in vitro experiments) and, if so, to analyze how this interaction occurs (in silico experiments). The results of these studies showed that direct interaction between some small- or medium-sized GSPE components and the enzyme is responsible for the increase in Cu/Zn-SOD activity.

KEYWORDS: Grape seed procyanidins extract; Cu/Zn-SOD; gene expression; diabetes; hepatocytes; oxidative stress; protein−ligand docking

INTRODUCTION

Flavonoids are a family of antioxidants that are largely found in fruits, vegetables, and popular beverages such as red wine in the form of procyanidins [a complex mixture of catechin oligomers whose average degree of polymerization is between 4 and 11 (*1*)]. Procyanidins are claimed to be anti-inflammatories and vasorelaxants, and to have positive effects on cardiovascular health (*2*). More specifically, the health-protective properties of procyanidins have been mainly attributed to their antioxidant activity, which involves mechanisms such as metal chelating and free radical scavenging (*3*). However, it has been recently reported that flavonoids do not act only as conventional hydrogen-donating antioxidants. They may also modulate cells by acting on protein kinase and lipid kinase signaling pathways (*4*). In particular, procyanidins have been shown to modulate, at concentrations that are achievable in plasma, the activity of regulatory enzymes such as cyclooxygenase, lipoxygenase, protein kinase C, angiotensin-converting enzyme, hyaluronidase, various forms of cytochrome P450, and the gene expression of SHP, CYP7A1, and cytokines (*5*−*7*).

It is widely accepted that an increase in oxidative stress takes part in the development and progression of diabetes (a group of metabolic diseases characterized by hyperglycemia, which is the result of defects in either insulin secretion, insulin action, or both) and its derived complications (*8*, *9*). Moreover, induced hyperglycemia increases glucose autoxidation and protein glycation, and the subsequent oxidative degradation of glycated protein leads to an enhanced production of reactive oxygen species (ROS) (*10*). There are also contradictory reports in the literature on how antioxidant enzymes are affected by diabetes-induced hyperglycemia. For example, they have been reported to decrease, increase, or remain unaltered in diabetic animals, and the age of the animal, the duration of the diabetes (*10*−*12*), and the tissues examined (*13*) can lead to considerable variability.

Superoxide anion radicals ($O_2^{\bullet -}$) are the major ROS generated in mitochondria. They are involved in producing several potentially damaging species (i.e., hydrogen peroxide,

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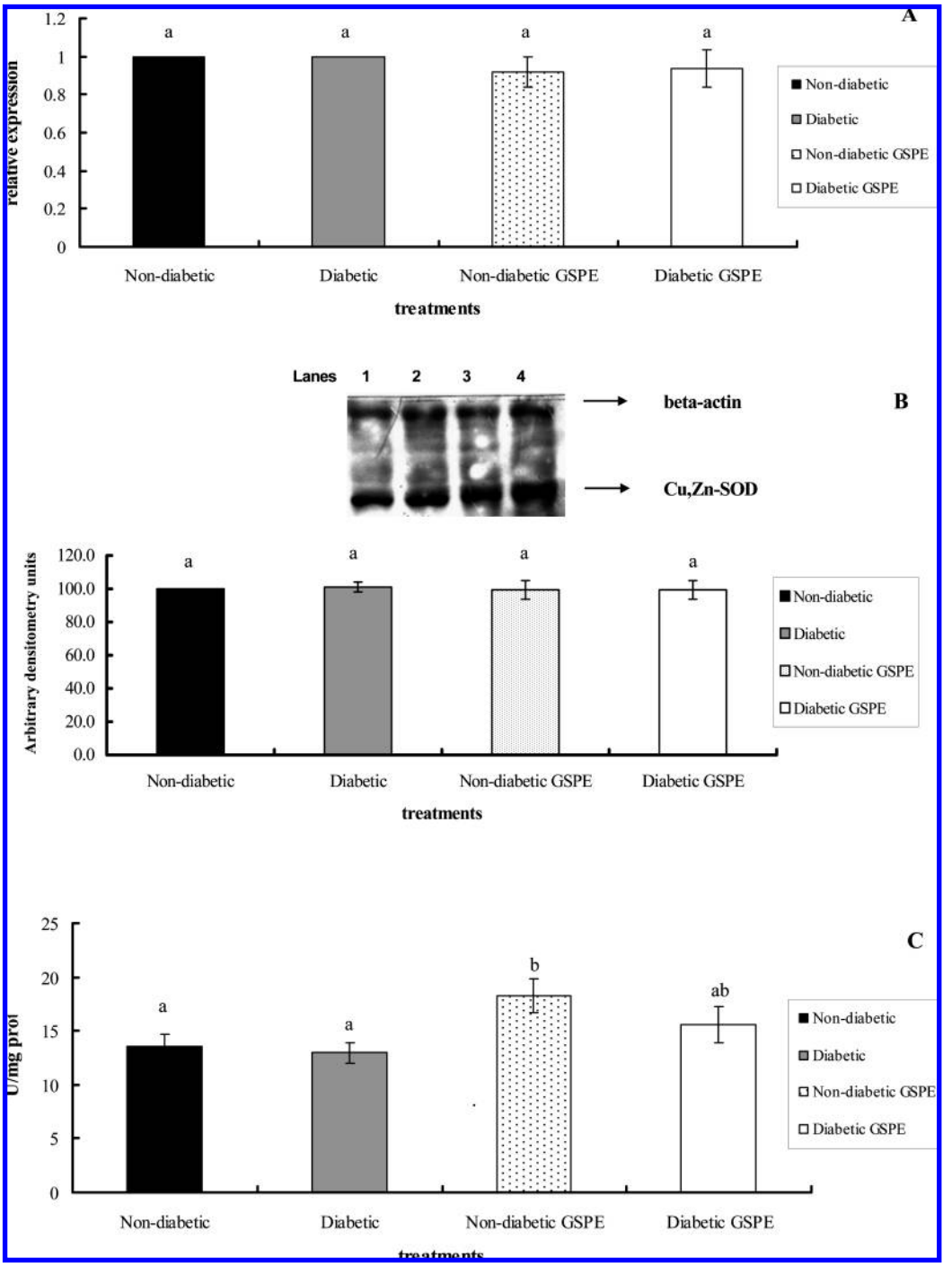

Figure 1. In vivo effect of GSPE treatment on Cu/Zn-SOD expression and activity. (**A**) Cu/Zn-SOD mRNA expression in vivo. Relative quantification by real-time PCR using GAPDH as the internal standard. Values are expressed as a percentage of the control (means ± SEM of four different experiments in triplicate). Significant differences ($p < 0.05$) were obtained with ANOVA and are indicated with superscripts (Scheffe's test). (**B**) Western analysis of Cu/Zn-SOD in rat's liver. Lanes: 1, nondiabetic rats; 2, diabetic rats; 3, nondiabetic GSPE rats; and 4, diabetic GSPE rats. Bars represent the arbitrary relative abundance of hepatic Cu/Zn-SOD vs β-Actin as the internal standard. Significant differences ($p < 0.05$) were obtained with ANOVA and are indicated with superscripts (Scheffe's test). (**c**) Cu/Zn-SOD enzyme activity in vivo. Values are expressed as a percentage of the control (means ± SEM of four different experiments in triplicate). Significant differences ($p < 0.05$) were obtained with ANOVA and are indicated with superscripts (Scheffe's test).

hydroxyl radicals, and peroxynitrite), which can damage cells by causing lipid peroxidation and oxidative damage in DNA and proteins (*14*). Therefore, the generation and/or removal of superoxide has been observed to play a significant role in a variety of critical homeostatic mechanisms at both the cellular and organism levels (*15*). Copper−zinc-superoxide dismutase (Cu/Zn-SOD) catalyzes the dismutation of the superoxide radical ($O_2^{\bullet-}$) into oxygen and hydrogen peroxide and, therefore, plays a key role in maintaining the above-mentioned homeostasis (*16*). Since biological macromolecules are a target for the damaging action of ROS, it would be very useful to understand how procyanidins affect Cu/Zn-SOD. So far, little work has been done to directly assess how procyanidins affect this enzyme. In previous studies, we have observed that moderate red wine consumption results in higher hepatic Cu/Zn-SOD activity in rats (*17*) and that a grape seed procyanidin extract (GSPE) improves the hepatic oxidative metabolism in vitro (*18*). Therefore, in the present study, we

first assess the effect of GSPE on the in vivo expression of Cu/Zn-SOD by using livers from streptozotocin-induced diabetic rats as an oxidative stress model. Then, we investigate how different doses of GSPE modulate in vitro mRNA expression, protein abundance, and the specific activity of Cu/Zn-SOD in rat Fao cell line hepatocytes. Finally, by using in vitro and in silico models, we show that at least some of the molecules in GSPE directly interact with Cu/Zn-SOD.

MATERIALS AND METHODS

Animal Experimental Procedures. Male Wistar rats weighing 250 g were purchased from Charles River (Barcelona, Spain). The animals were housed in animal quarters at 22 °C with a 12 h light/dark cycle and were fed ad libitum. The Animal Ethics Committee of our university approved all of the procedures. Type-1 diabetes mellitus was induced by intraperitoneal injection of a freshly prepared solution of streptozotocin (STZ) 70 mg/kg in 50 mM citrate buffer, pH 4.5. The only diabetic animals used were those with polyuria, glycosuria, and hyperglycemia (≈ 20 mM) 2−3 days postinduction. All studies were carried out 1 week after STZ had been injected.

Rats were divided into four groups of 6−7 rats each. (1) Nondiabetic group: rats were fed an oral gavage with vehicle (tap water). (2) Nondiabetic GSPE group: rats were given an oral gavage of GSPE (i.e., Vitaflavan from Les Dérives Résiniques et Térpeniques, Dax, France) consisting of 16.5% monomers, 18.7% dimers, 16% trimers, 9.3% tetramers, 4.2% phenolic acids, and 35.5% higher polymers (molecular weight = 1575 g/mol). An aqueous solution of 2 g/20 mL of GSPE was prepared and administered at a dose of 250 mg/kg body weight. (3) Diabetic group: rats were fed an oral gavage with vehicle (tap water). (4) Diabetic GSPE group: rats were fed an oral GSPE gavage (250 mg/kg body weight). The procyanidin dose used is one-fifth of the NOAEL (no-observed-adverse-effect level) described for GSPE and male rats (*19*).

After 5 h of GSPE gavage, the rats were beheaded, and their livers were excised, immediately frozen in liquid nitrogen, and stored at −80 °C.

Cell Line and Culture. Fao cells were routinely cultured in F-12 Coon's modification medium (Sigma), supplemented with 5% fetal bovine serum and 1% penicillin/streptomycin (all of which were provided by BioWhittaker). Cells were grown at 37 °C in a humidified atmosphere of 5% CO_2. They were seeded 3−4 days before use in 6-well plates (Corning) at a density of (4−5) × 10^5 cells/well. Cells were incubated with GSPE at concentrations between 0 and 150 mg/L for 24 h.

Table 1. In Vivo Analysis of Cu/Zn-SOD Gene Expression Ratios[a]

groups	activity/protein	protein/mRNA
nondiabetic	100	100
diabetic	99	100
nondiabetic GSPE	133	110
diabetic GSPE	115	106

[a] Gene expression in Cu/Zn-SOD under different doses of GSPE. mRNA, protein, and activity levels found in control animals are regarded as 100%. The values of the different treatments are presented as percentages of the control value.

Cytotoxicity. The cytotoxicity of GSPE was evaluated with the lactate dehydrogenase (LDH) assay. It was determined spectrophotometrically by the rate of NADH utilization in the enzyme-catalyzed back reaction of pyruvate conversion to lactate using the LDH kit (QCA, Barcelona, Spain). By relating LDH leakage to total LDH activity after the hepatocytes had been lysed, we evaluated the effect that GSPE had on cell viability.

Superoxide Dismutase Enzyme Activity. Liver tissue was homogenized in 0.1 M sodium phosphate buffer at pH 7.4 (1:10 wt/vol) and centrifuged at 20000*g* for 10 min, and the supernatant was collected. All of these steps were performed at 4 °C. After an aliquot had been saved for the protein assay, the rest of the supernatant was used to measure Cu/Zn-SOD activity by quantifying the inhibition of pyrogallol autoxidation (*20*) at 420 nm for 3 min.

Fao cells were scraped into 50 mM phosphate buffered saline (PBS) at pH 7.4 containing 0.1% Triton X-100 and then disrupted by sonication for 15 s. After centrifugation at 10.000*g* for 10 min at 4 °C, the supernatant was collected for protein determination and the analysis of Cu/Zn-SOD activity. Thus, the Cu, Zn-SOD activity was determined by measuring the extent to which the epinephrine autoxidation caused by the superoxide anion had been inhibited at 480 nm and 37 °C (*21*). The reaction mixture contained 50 mM carbonate buffer at pH 10.2, 1 mM EDTA, 50 μL of cellular extract and 5 mM epinephrine. The reaction was monitored for 4 min.

Determination of Total Protein Content. The total protein content in the samples was measured by the Bradford method (*22*). Bovine serum albumin was used as the standard, and the absorbance was measured at 595 nm.

Western Blot Analysis. After GSPE treatment, the liver was homogenized, and hepatocytes were resuspended and lysed in 1 mL of the following buffer: 1× PBS, 1% nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS. To inhibit proteases, 5 mL of a buffer containing 25 μL of 2 mM leupeptine (Sigma), 25 μL of 2 mM pepstatine B (Sigma), and 100 μL of 0.1 M PMSF (Sigma) was added.

Samples containing 7.5 μg of total protein were electrophoresed on SDS−polyacrylamide gel (12%), using the Laemmli method (*23*). At the same time, molecular weight standards were loaded into separate lanes. The proteins were transferred onto a nitrocellulose membrane (Protran, Schleicher & Schuell, Germany). Blots were then probed with rabbit Cu/Zn-SOD polyclonal antibody, with β-Actin antibody as the internal standard (Stressgen Biotechnologies) and then with peroxidase-labeled secondary antibody and the chemiluminescent substrate luminol (ECL Western Blotting, Amersham Pharmacia Biotech). Chemiluminescent detection of specifically labeled protein was quantified by using Quantity One software (Bio-Rad) to measure the density of the bands detected.

RNA Isolation and Real Time RT-PCR. Total RNA was isolated from rat livers or Fao cells using the High Pure Isolation kit (Roche). The amount of total RNA was estimated by optical density

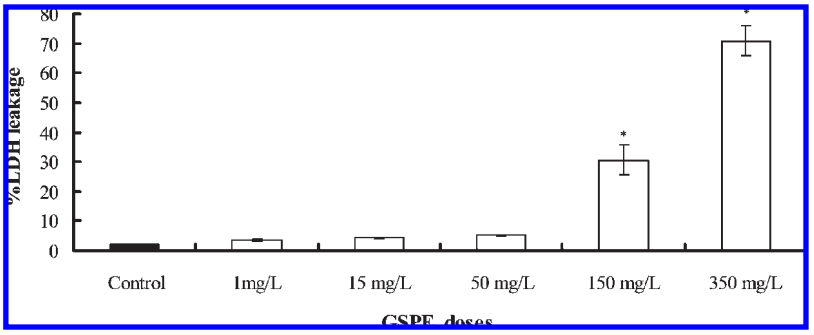

Figure 2. Viability of Fao cells after treatment with different doses of GSPE for 24 h. Cell viability was assessed by LDH assay. Values are expressed as the percentage of LDH leakage. Experimental values are the mean ± SEM of three different experiments in triplicate. *Significant differences ($P < 0.05$) vs control value were determined using a *t*-test.

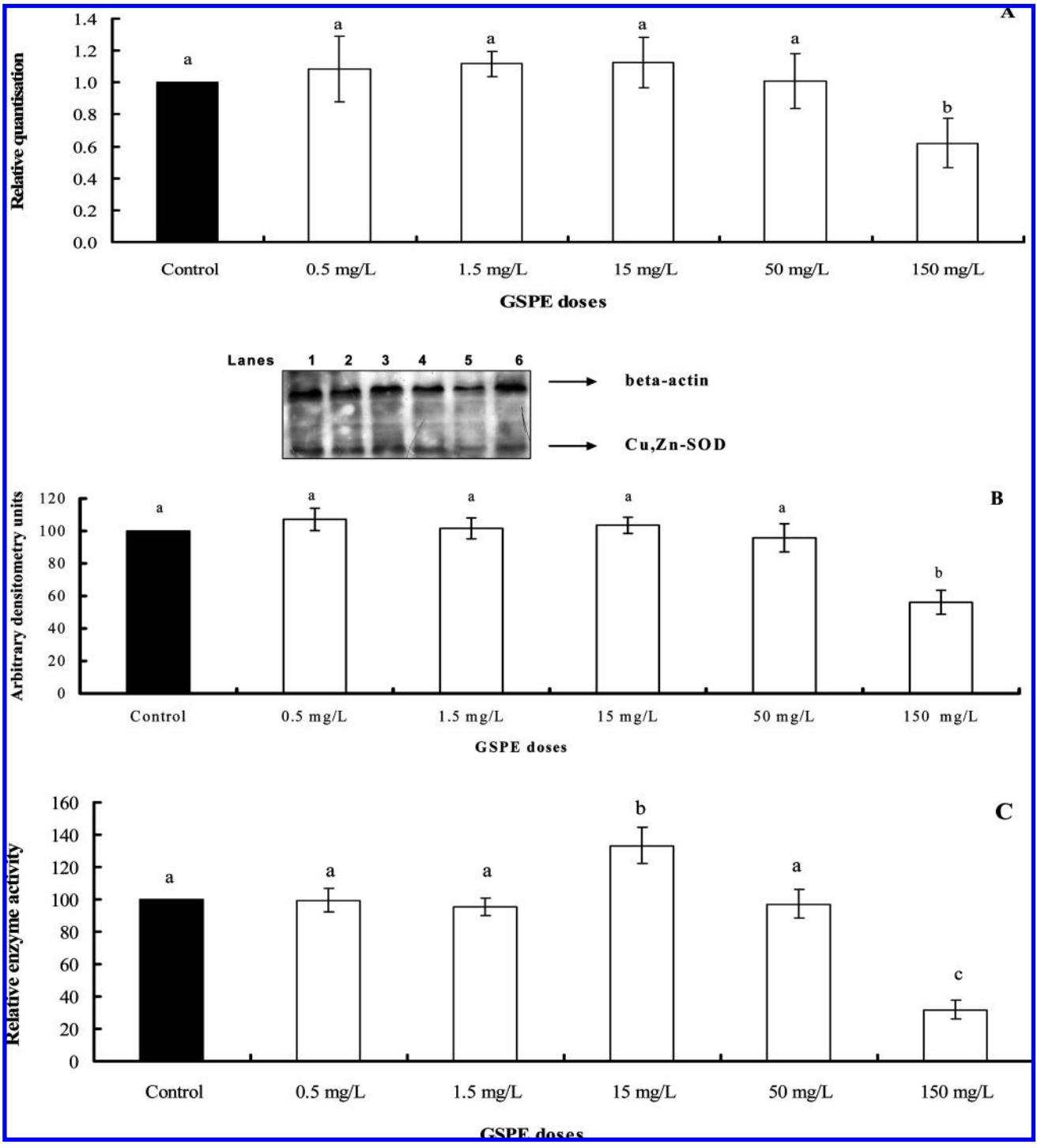

Figure 3. In vitro analysis of the effect of GSPE on Cu/Zn-SOD expression and activity. (**A**) Cu/Zn-SOD mRNA expression in Fao cells under different doses of GSPE exposure. Relative quantification by real time PCR using GAPDH as internal standard after reverse transcription. Values are expressed as a percentage of control (means ± SEM of four different experiments in triplicate). Significant differences ($p < 0.05$) were obtained with ANOVA and are indicated with superscripts (Scheffe's test). (**B**) Western analysis of Cu/Zn-SOD. Lanes: 1, control; 2, 0.5 mg/L; 3, 1.5 mg/L; 4, 15 mg/L; 5, 50 mg/L; 6, 150 mg/L. Bars represent the arbitrary densitometry abundance of Cu/Zn-SOD for each treatment condition. Results are shown as the mean ± SEM ($n = 4$). Significant differences ($p < 0.05$) were obtained with ANOVA and are indicated with superscripts (Scheffe's test). (**C**) Cu/Zn-SOD enzyme activity. Values are expressed as a percentage of the control (means ± SEM of four different experiments in triplicate). Significant differences ($p < 0.05$) were obtained with ANOVA and are indicated with superscripts (Scheffe's test).

at 260 nm. One microgram of RNA was transcribed into cDNA with SuperScript-II (Life Technologies). Cu/Zn-SOD and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were studied by amplifying the reversibly transcribed RNAs with specific primer pairs (from Applied Biosystems, Warrington, UK), as follows: Cu/Zn-SOD sense primer, 5′-AGC GGA TGA AGA GAG GCA TGT-3′; antisense primer, 5′-CAC ACG ATC TTC AAT GGA CAA T-3′; GAPDH sense primer, 5′-TGC CAA GTA TGA TGA CAT CAA GAA-3′; antisense primer, 5′-AGC CCA GGA TGC CCT TTA GT-3′. Primers were added at a final concentration of 0.3 μM to a 25 μL reaction mixture containing 10 ng of cDNA and 5X Sybr Green (Applied Biosystems).

In accordance with the manufacturer's instructions (Applied Biosystems), the mixture was incubated at 50 °C for 2 min and

activated for 2 min at 95 °C. Then both genes were subjected to 40 cycles of sequential steps in a thermal cycler for initial melting (15 s at 95 °C) and an annealing/extension at 60 °C for 2 min.

Cu/Zn-SOD and GAPDH mRNA levels were measured in a fluorescent thermal cycler (GeneAmp 5700 Sequence Detection System, Applied Biosystems). The level of Cu/Zn-SOD mRNA was normalized to the level of GAPDH mRNA detected in each sample.

GSPE Direct Interaction with Cu/Zn-SOD Analysis. Commercial Cu/Zn-SOD (S8160 Sigma-Aldrich) was dissolved in 0.1 M sodium phosphate buffer (pH 7.4) until 100 U/mL. This solution was incubated for a maximum of 24 h with 1.5 mg/L GSPE, and aliquots were removed from the reaction vials at different times (0, 30, 90, 240, and 1440 min, respectively). Then, the activity was measured by the pyrogallol assay (as has been previously described) in aliquots containing 15 U/mL of Cu/Zn-SOD either preincubated or not with GSPE. In order to discount the possible interference of GSPE in pyrogallol autoxidation, assays with GSPE and pyrogallol but without the enzyme were also performed.

In Silico Studies. In order to study whether some of the molecules in GSPE (or their metabolites) were able to bind to Cu/Zn-SOD, we performed a protein−ligand docking assay. This assay was done by applying a blind-docking protocol that analyzed if these molecules were able to bind with high affinity to any part of the structure of Cu/Zn-SOD. To carry out this in silico assay, we used (a) our in-house database of phenolic compound structures as a ligand source; (b) the Cu/Zn-SOD structure that has been deposited in the PDB (http://www.pdb.org) with code 2c9v; and (c) the software programs BDT (http://www.quimica.urv.cat/~pujadas/BDT/) and AutoDock (http://autodock.scripps.edu/).

Statistical Analysis. Results are expressed as the mean ± SEM. Significance was tested by a *t*-test or one-way ANOVA (SigmaStat Version 10.0 for Windows, SPSS, Richmond, CA). We used Scheffe's test of honestly significant differences to make pairwise comparisons ($P < 0.05$ were considered statistically significant).

Table 2. In Vitro Analysis of Cu/Zn-SOD Gene Expression Ratios under Different Doses of GSPE[a]

GSPE doses	activity/protein	protein/mRNA
control	100	100
0.5 mg/L	93	99
1.5 mg/L	94	106
15 mg/L	129	92
50 mg/L	102	95
150 mg/L	57	91

[a] Gene expression in Cu/Zn-superoxide dismutase under different doses of GSPE. mRNA, protein, and activity levels found in control cells are regarded as 100%. The values of treatments are presented as percentages of the control value.

RESULTS

Expression Profile of Cu/Zn-SOD: Effect of GSPE on Livers from Streptozotocin-Induced Diabetic Rats. Figure 1A−C shows the effect of GSPE on mRNA levels, the amount of protein, and the enzyme activity of Cu/Zn-SOD in both diabetic and nondiabetic rats. **Figure 1A** and **B** shows that there were no differences in the Cu/Zn-SOD mRNA levels and in the amount of protein among the different treatments. However, as can be seen in **Figure 1C**, Cu/Zn-SOD activity increased significantly in the nondiabetic GSPE group (+20%) relative to the nondiabetic group, whereas it only increased slightly in the diabetic GSPE group relative to the diabetic group. We did not find statistical differences between the diabetic or nondiabetic groups (see **Figure 1C**). Moreover, as illustrated in **Table 1**, the activity/protein and protein/mRNA ratio values were different only under GSPE exposure.

In Vitro Cytotoxicity. Figure 2 shows LDH leakage into the extracellular media of the Fao cells, which were incubated with several doses of GSPE for 24 h. The LDH leakage increased in a dose-dependent manner. Doses above 150 mg/L presented a high percentage of LDH leakage (>40%) in the culture medium; therefore, they were not used for subsequent experiments. Taking into account these results on cell viability, we considered 150 mg/L GSPE as the reference of an excessive GSPE dose in Cu/Zn-SOD expression.

Expression Profile of Cu/Zn-SOD in Fao Cells: Effect of GSPE Treatments. Figure 3A−C shows the effect of GSPE on mRNA levels, the amount of protein, and the enzyme activities of Cu/Zn-SOD in Fao cells. **Figure 3A** shows that Cu/Zn-SOD mRNA expression was not affected by GSPE

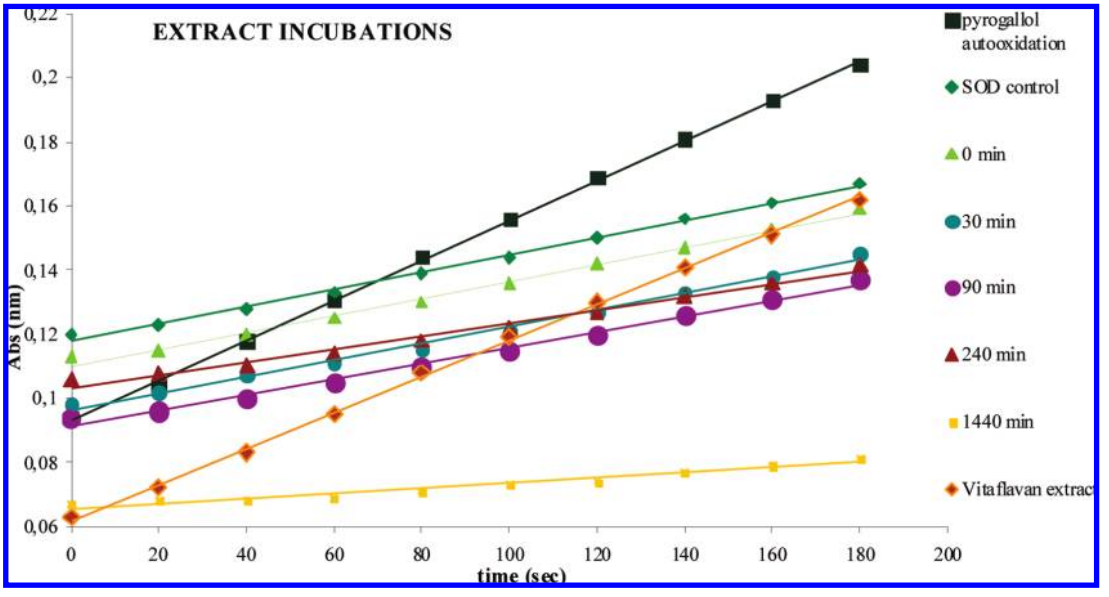

Figure 4. Inhibition of the autoxidation of pyrogallol by Cu/Zn-SOD after different coincubations of the enzyme with GSPE. The pyrogallol autoxidation line shows its speed of self-oxidation in the absence of Cu/Zn-SOD. The Vitaflavan line shows the autoxidation of pyrogallol when it is incubated with GSPE but in the absence of the enzyme. The SOD control line shows the autoxidation of pyrogallol when it is incubated with the enzyme but in the absence of GSPE. The remaining lines show the autoxidation of pyrogallol when it is incubated with the enzyme (which has been coincubated with GSPE for different incubation times).

Table 3. In Silico Results for the Binding of the Phenolic Compounds Most Frequently Found in Grape Seed Extracts (and Their Metabolites) to Cu/Zn-SOD[a]

phenolic acids	stilbenes
sinapic acid	*trans*-resveratrol
gallic acid	*trans*-resveratrol 3-glucuronide §
4-*O*-methylgallic acid§‡	*trans*-resveratrol 4′glucuronide §
gallic 3-glucuronide acid§†	*trans*-resveratrol 3-sulfate *
gallic 4-glucuronide acid §†	
protocatechuic acid	
syringic acid	
vanillic acid	

flavanol monomers (catechins)	flavanol procyanidins (dimers and trimers)	
(+)-catechin	B1	(−)-epicatechin-(4β→8)-(+)-catechin
(+)-catechin 3′-glucuronide *†	B2	***(−)-epicatechin-(4β→8)-(−)-epicatechin***
(+)-catechin 3-glucuronide*†	B3	(+)-catechin-(4α→8)-(+)-catechin
(+)-catechin 4′-glucuronide*†	B4	(+)-catechin-(4α→8)-(−)-epicatechin
(+)-catechin 7-glucuronide*†	B5	(−)-epicatechin-(4β→6)-(−)-epicatechin
3′-*O*-methyl-(+)-catechin*	B6	(+)-catechin-(4α→6)-(+)-catechin
3′-*O*-methyl-(+)-catechin 3-glucuronide*†	B7	(−)-epicatechin-(4β→6)-(+)-catechin
3′-*O*-methyl-(+)-catechin 4′-glucuronide*†	B8	(+)-catechin-(4α→6)-(−)-epicatechin
3′-*O*-methyl-(+)-catechin 7-glucuronide*†	C1	(−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(−)-epicatechin
(−)-epicatechin	T2	(−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(+)-catechin
(−)-epigallocatechin		
(−)-epicatechin 3-gallate		
(−)-epicatechin 3′-glucuronide*†		
(−)-epicatechin 3-glucuronide*†		
(−)-epicatechin 4′-glucuronide*†		
(−)-epicatechin 7-glucuronide*†		
4′-*O*-methyl-(−)-epicatechin§		
4′-O-methyl-(−)-epicatechin 3-glucuronide§		
4′-*O*-methyl-(−)-epicatechin 5-glucuronide§		
4′-O-methyl-(−)-epicatechin 7-glucuronide§		
3′-*O*-methyl-(−)-epicatechin 7-glucuronide§		
4′-*O*-methyl-(−)-epigallocatechin§		
(−)-epigallocatechingallate (EGCG)		
(−)-catechin 3-gallate		

[a] This table shows the phenolic compounds most frequently found in grape seed extracts and the derived metabolites found in human (§) or rat (*) plasma or urine. Metabolites produced by intestinal microflora are also indicated by the symbol (‡). Several studies have detected glucuronide compounds in plasma or urine, but the exact site of glucuronidation has yet to be determined (thererefore, we have built all the possible structures for these conjugated compounds (†)). The abbreviations *Glcde*, *Glc* and *Gal* are for glucuronide, glucose, and gallate, respectively. The phenolic compounds that are predicted to bind to Cu/Zn-SOD are shown in bold and italics.

exposure except at 150 mg/L when it decreased significantly (−30%) in relation to the control value. **Figure 3B** shows Western Blot detection for Cu/Zn-SOD and the densitometric data relative to β-Actin. The pattern was the same as for mRNA levels; therefore, the amount of protein did not present significant changes except at 150 mg/L, where the relative amount of Cu/Zn-SOD was also significantly diminished (by about 50%). It is interesting to see that the determinations of enzyme activity show that the levels of Cu/Zn-SOD were significantly enhanced at 15 mg/L (+ 33%; see **Figure 3C**), which was not observed in either mRNA or protein profiles. At 150 mg/L, however, the enzyme activity decreased by 70%, just as it did in the mRNA and protein profiles.

Therefore, in order to identify more easily the step and dose of GSPE at which the Cu/Zn-SOD regulation was greatest, we derived the protein/mRNA and activity/protein ratios (**Table 2**). The most important changes relative to the control situation were in the activity/protein ratio at both 15 mg/L and 150 mg/L GSPE.

Effect of Incubation with GSPE on Cu/Zn-SOD Activity. The results in **Figures 4** and **5** show that Cu/Zn-SOD activity increases on incubation time with GSPE. Thus, after 90 or 1440 min of coincubation of Cu/Zn-SOD with GSPE, the enzyme activity relative to no coincubation (SOD control and 0 min lines) increases 1.33- and 1.73-fold, respectively. **Figure 4** also shows that, in the absence of the enzyme, GSPE cannot inhibit pyrogallol autoxidation (i.e., the slopes of the pyrogallol autoxidation and Vitaflavan lines are the same). These results indicate that the increase in enzyme activity upon coincubation with GSPE is the result of the formation of a complex between Cu/Zn-SOD and at least some of the molecules present in GSPE.

Docking of Molecules in GSPE on Cu/Zn-SOD. In order to analyze which of the molecules in GSPE can bind to Cu/Zn-SOD and increase its activity, we carried out a protein−ligand docking experiment. During this in silico experiment, the ligands were the chemical structures of the molecules that are found in grape seeds and their derived metabolites. Docking results show that (1) only some of the ligands tested [i.e., protocatechuic acid, syringic acid, (−)-epicatechin, (−)-epigallocatechin, 4′-*O*-methyl-(−)-epicatechin 3-glucuronide, 4′-*O*-methyl-(−)-epicatechin 7-glucuronide, and procyanidin B2] are able to bind to the enzyme (see **Table 3**) and that (2) there is a single binding site located near the active site of each homodimer subunit (see **Figure 6**).

DISCUSSION

It has been reported that the pathology of diabetes involves high oxidative stress because hyperglycemia depletes the activity of the antioxidative defense systems and thus promotes the generation of free radicals (*24*). Additionally, the concerted actions of antioxidant enzymes, which keep the concentration of free radicals in cells relatively low, are overwhelmed in many diseases correlated with a cellular oxidative stress status. Our article, then, focuses on procyanidins because we have previously shown that their administration (a) has an antihyperglycemic effect on streptozotocin-induced diabetic rats (*25*); (b) stimulates glucose uptake in L6E9 myotubes and 3T3-L1 adipocytes by shearing the PI3K and p38 MAPK insulin signaling pathways and, in consequence, stimulates GLUT-4 translocation to the plasma membrane (*25*); (c) improves the atherosclerotic risk index (*6*, *7*); (d) induces liver CYP7A1 and SHP expression in healthy rats (*6*); (e) interferes with adipogenesis of 3T3-L1 adipocytes at the onset of differentiation (*26*); (f) lowers triglyceride levels signaling through SHP (*27*); (g) enhances bile acid-activated FXR activity in vitro, and reduces triglyceridemia in vivo in an FXR-dependent manner (*28*); (h) activates hepatic antioxidant enzymes in rats that consume moderate amounts of

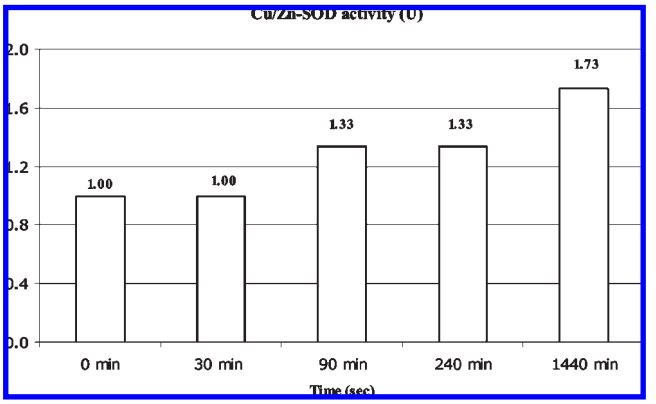

Figure 5. Effect of the different coincubations of Cu/Zn-SOD with GSPE on enzyme activity. The Figure shows how the different coincubation conditions increase the enzyme activity relative to no coincubation with GSPE (where 1U for Cu/Zn-SOD corresponds to a 50% of inhibition of pyrogallol autoxidation) (*20*).

red wine (*17*); and (i) improves the hepatic oxidative metabolism and antigenotoxic effects on Fao cells submitted to H_2O_2 (*18*, *29*). Therefore, in the current study we examined the effect of GSPE on the mRNA level, the amount of protein, and the activity of Cu/Zn-SOD in two models: (1) streptozotocin-induced diabetes in Wistar rats as an in vivo model for oxidative stress and (2) hepatocarcinoma Fao cells as an in vitro model.

There are discrepancies in the literature about the regulation of Cu/Zn-SOD in diabetes (*10*, *13*, *30*, *31*). In diabetic animals, we found no differences in Cu/Zn-SOD expression and a slight but not significant decrease in Cu/Zn-SOD activity. These results are in agreement with the findings of Reddi et al. and Godin et al. (*13*, *30*), who reported that Cu/Zn-SOD activity decreased in the kidney, heart, and liver of diabetic rats (they did not examine the mRNA and protein levels). However, they are in disagreement with the findings of Kakkar and Hammes et al. (*10*, *31*), who reported an increase in Cu/Zn-SOD activity in the liver, heart, and pancreas and no change in activity in the kidney of diabetic rats. Besides this, the increased Cu/Zn-SOD activity in diabetic GSPE rats that we observed is not statistically different from the activity in diabetic rats, although it is at the limit of the significance level. At this point, it should be pointed out that Maritim et al. reported not only that diabetes did not affect Cu/Zn-SOD activity in any tissue but also that there was a slight increase in its activity when the livers of diabetic rats were treated with pycnogenol (a procyanidin extract from maritime pine bark) (*32*). Similar observations have been reported with melatonin and gemfibrozil (*32*, *33*). Furthermore, our results on the in vivo enzyme activity/protein and protein/mRNA ratios confirm that Cu/Zn-SOD is post-translationally regulated by GSPE intake in both nondiabetic and diabetic rats.

Therefore, in order to assess how GSPE treatment can modulate Cu/Zn-SOD regulation and to determine the specificity of its in vitro response, we incubated Fao cells with different GSPE doses in order to (1) confirm the Cu/Zn-SOD kind of regulation that was observed in vivo; (2) find whether the GSPE effect was dose-dependent; and (3) find the GSPE dose that produces the greatest effect. It is well known that the liver and the small intestine are the main organs in which flavonoids undergo extensive phase II metabolism. Therefore, as Fao cells have the enzymatic machinery required to derivatize flavonoids, we were able to assess the effect of procyanidins by directly adding their native form to the culture medium (*34*). The exposure of these cells to GSPE did not

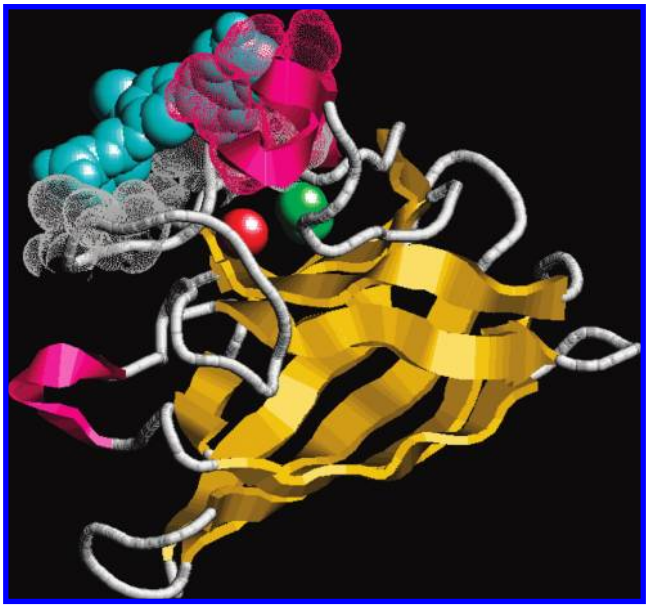

Figure 6. Protein−ligand docking results. The figure shows the location at which some of the molecules in grape seed extracts and derived metabolites are predicted to bind to one of the subunits of the Cu/Zn-SOD homodimer. This in silico experiment was carried out with no a priori restriction on where the ligands can bind to the enzyme (i.e., the whole protein surface was analyzed for putative binding sites). The protein is shown in ribbon form and color coded according to the secondary structure (i.e., magenta, yellow and white for helices, β-strands, and β-turns/loops, respectively). The metabolite 4′-*O*-methyl-(−)-epicatechin 3 glucuronide is shown in cyan and in spacefill format. The proteic area 5.0 Å around any 4′-*O*-methyl-(−)-epicatechin 3 glucuronide atom is shown with dots so that the protein−ligand contacts can be seen better. Copper and zinc ions are shown as green and red spheres, respectively. The molecular visualization software used is RasMol (http://www.openrasmol.org/) (*40*).

alter the Cu/Zn-SOD mRNA levels or the amount of protein, but the enzyme activity was significantly enhanced at 15 mg/L, and GSPE was observed to have no dose-dependent effect on Cu/Zn-SOD activity. At this point, it is interesting to point out that the literature has attributed beneficial effects to the 15 mg/L GSPE dose. For example, Pinent et al. reported that 15 mg/L GSPE significantly stimulates the glucose uptake in L6E9 (*25*), and Roig et al. and Llópiz et al. showed that the hepatic enzyme metabolism and antigenotoxic effects improved under oxidative stress (*18*, *29*). It is also interesting to point out that, in terms of molar concentration and considering the average molecular weight of GSPE (see the Materials and Methods section), 15 mg/L is equivalent to a 9−10 μM concentration, which is physiologically attainable in plasma according to Kroon et al (*34*).

Many authors have reported that the differences in the basal and inducible mRNA expression levels of Cu/Zn-SOD may be dependent on the cell type studied (*35*, *36*). This is in agreement with Kameoka et al. (*37*) who reported no changes in the Cu/Zn-SOD expression pattern after isoflavonoid daidzein treatment in Caco-2 cells. When we compared the hepatic Cu/Zn-SOD expression profile, both in vivo and in vitro (at 15 mg/L GSPE) we found that the enzyme activity/protein ratios were similar in both models because the enzyme activities are increased under GSPE treatments. This common expression profile reinforces a probably post-translational Cu/Zn-SOD regulation by GSPE. However, the considerable decrease in Cu/Zn-SOD expression at 150 mg/L GSPE enabled us to take this dose as the negative control for evaluating GSPE

effects on Cu/Zn-SOD expression. Since LDH results (see **Figure 2**) showed an increase in cellular toxicity, the mRNA levels, amount of protein, and enzyme activity decreased significantly. This is probably due to protein oxidation (*38*) and mRNA fragility, and confirms Skibola's findings that a slightly pro-oxidant effect at high doses of flavonoids can modify the redox status of the cell by altering the antioxidant mRNA binding protein, or affecting the mRNA stability and thus, indirectly, enzyme synthesis and its activity (*39*).

Therefore, in response to these results, we investigated whether any of the molecules in GSPE can directly bind to Cu/Zn-SOD and cause the observed increase in the activity of the enzyme. We incubated commercial Cu/Zn-SOD with GSPE and confirmed that the enzyme activity increases with incubation time, which indicates that the direct interaction between some molecules in GSPE and Cu/Zn-SOD is directly related to the observed increase in activity. Therefore, by using in silico methodologies we predicted (1) what the probable ligand-binding site for these molecules in Cu/Zn-SOD is and (2) which of the molecules present in GSPE (and resulting metabolites) can bind to the enzyme. Our results showed that (1) only some of these molecules can bind to Cu/Zn-SOD and, therefore, activate it and (2) there is only one probable binding site located close to the active site of each subunit in the dimer.

To sum up, although mRNA and protein levels did not increase, the Cu/Zn-SOD activity was significantly stimulated at 15 mg/L of GSPE treatment, which strongly suggests a post-translational regulation of Cu/Zn-SOD under GSPE exposure. This confirms our in vivo results in an oxidative stress model such as diabetes. We have also confirmed that the changes in Cu/Zn-SOD activity are attributable to direct interaction between molecules in GSPE and the enzyme, and we have found its probable binding site on the Cu/Zn-SOD molecule by in silico studies. Nevertheless, further experiments will be necessary if the role of GSPE in the molecular mechanisms underlying this regulation is to be determined.

ABBREVIATIONS USED

Cu/Zn-SOD, copper,zinc-superoxide dismutase; LDH, lactate dehydrogenase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GSPE, grape seed procyanidin extract; ROS, reactive oxygen species; STZ, streptozotocin.

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Supporting Information Available: The chemical structure of the molecules used during the in silico assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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