ACTA ABP BIOCHIMICA POLONICA

Vol. 58, No 1/2011
45–50
on-line at: www.actabp.pl

Regular paper

Effects of olive leaf polyphenols against H_2O_2 toxicity in insulin secreting β-cells

Ahmet Cumaoğlu[1], Lucia Rackova[2], Milan Stefek[2], Murat Kartal[3], Pierre Maechler[4] and Çimen Karasu[5]✉

[1]Gazi University, Faculty of Medicine, Department of Medical Biochemistry, Ankara, Turkey; [2]Institute of Experimental Pharmacology and Toxicology, Slovak Academy of Sciences, Bratislava, Slovak Republic; [3]Ankara University, Faculty of Pharmacy, Department of Pharmacognosy, Ankara, Turkey; [4]Geneva University, Faculty of Medicine, Department of Cell Physiology and Metabolism, Geneva, Switzerland; [5]Gazi University, Faculty of Medicine, Department of Medical Pharmacology, Ankara, Turkey.

In pancreatic β-cells, although H_2O_2 is a metabolic signal for glucose stimulated insulin secretion, it may induce injury in the presence of increased oxidative stress (OS) as in the case of diabetic chronic hyperglycemia. *Olea europea* L. (olive) leaves contain polyphenolic compounds that may protect insulin-secreting cells against OS. The major polyphenolic compound in ethanolic olive leaf extract (OLE) is oleuropein (about 20%), thus we compared the effects of OLE with the effects of standard oleuropein on INS-1 cells. The cells were incubated with increasing concentrations of OLE or oleuropein for 24 h followed by exposure to H_2O_2 (0.035 mM) for 45 min. H_2O_2 alone resulted in a significantly decreased viability (MTT assay), depressed glucose-stimulated insulin secretion, increased apoptotic and necrotic cell death (AO/EB staining), inhibited glutathione peroxidase activity (GPx) and stimulated catalase activity that were associated with increased intracellular generation of reactive oxygen species (ROS) (fluorescence DCF). OLE and oleuropein partly improved the viability, attenuated necrotic and apoptotic death, inhibited the ROS generation and improved insulin secretion in H_2O_2-exposed cells. The effects of oleuropein on insulin secretion were more pronounced than those of OLE, while OLE exerted a stronger anti-cytotoxic effect than oleuropein. Unlike OLE, oleuropein had no significant preserving effect on GPx; however, both compounds stimulated the activity of catalase in H_2O_2-exposed cells. These findings indicate different modulatory roles of polyphenolic constituents of olive leaves on redox homeostasis that may have a role in the maintenance of β-cell physiology against OS.

Keywords: olive, polyphenol, oleuropein, hydrogen peroxide, insulin, β-cells, glucose, apoptosis, oxidative stress

Received: 08 July, 2010; revised: 03 November, 2010; accepted: 18 December, 2010; available on-line: 07 March, 2011

INTRODUCTION

Pancreatic β-cells secrete insulin to regulate blood glucose homeostasis. The dysfunction and injury of these cells contribute to the pathogenesis of type 1 and type 2 diabetes (Ihara *et al.*, 1999; Cnop *et al.*, 2005; Del Guerra *et al.*, 2005; Kaneto *et al.*, 2007). Insulin secretion is subject to control by nutrients and by hormonal, neural, and pharmacological factors. Among these, glucose is by far the most important regulator of the machinery of insulin secretion (Gembal *et al.*, 1992). It has been well documented that glycolytic and oxidative events coupled with the generation of reactive oxygen species (ROS) lead to accelerated ATP generation and are key transduction phenomena in β-cell signaling (Pi *et al.*, 2007). However, one of the unique features of β-cells is their relatively low expression of H_2O_2-detoxifying antioxidant enzymes such as glutathione peroxidase (GPx) (Kaneto *et al.*, 2007), which could render β-cells susceptible to oxidative damage. Although H_2O_2 has emerged as a physiological mediator of insulin secretion (Merglen *et al.*, 2004), chronic hyperglycemia has been shown to be involved in β-cell dysfunction, a phenomenon known as glucotoxicity (Robertson *et al.*, 2003; Robertson & Harmon, 2006). The multiple biochemical pathways and mechanisms of the glucose toxicity have in common the formation of ROS, which in excess and over time can cause chronic oxidative stress (OS) (Robertson *et al.*, 2003; Robertson & Harmon, 2006). Oxidative stress plays a central role in cellular injury and cell death, and the cellular responses to OS involve changes in mitochondria and other organelles, notably endoplasmic reticulum, and can lead to a number of cell death modes, which cover a spectrum from apoptosis to necrosis and include autophagy. Apoptosis has been shown to be the main way of β-cell death in the presence of chronic hyperglycemia and OS (Eizirik *et al.*, 2001; Rhodes, 2005). Therefore, the protection of β-cells against OS-induced injury and dysfunction is important in the prevention of diabetes. As in the case of studies on the retardation of diabetes-induced complications by antioxidants (Güneş *et al.*, 2005; Yülek *et al.*, 2007), great attention is now focused on natural antioxidants in the protection of β-cells against OS-induced injury.

Olive (*Olea europaea* L.) leaves and fruits contain several groups of constituents, including polyphenols, flavonoids, flavones, iridoids and carbohydrates (Gariboldi *et al.*, 1986; Heimler *et al.*, 1992; Romani *et al.*, 1994). These substances have a considerable pharmacological action including antioxidant activity and low toxicity (Soni *et al.*, 2006). Oleuropein, a phenolic secoiridoide, is used as a well known compound of extracts and its concentration is significantly high in leaves and fruit. Oleuropein has a high antioxidant activity *in vitro* (Speroni *et al.*, 1998), scavenges superoxide anions, hydroxyl radicals, and hypochlorous acid-derived radicals (Visioli *et al.*, 1998). Ol-

✉e-mail: karasu@gazi.edu.tr

Abbreviations: AO/EB, acridine orange/ ethidium bromide staining; GPx, glutathione peroxidase; MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide; OLE, olive leaf extract; OS, oxidative stress

ive leaves have an ethnomedical usage in the management of diabetes, and the anti-diabetic effect of olive leaves or oleuropein has already been demonstrated in animal models (Gonzalez *et al.*, 1992; Jemai *et al.*, 2009; Eidi *et al.*, 2009). However, the cellular mechanism(s) of their effects responsible for blood glucose control, including the effects on insulin secretion and any relation with cellular redox state have not been studied yet in insulin secreting cells. Thus, we investigated the effects of polyphenolic olive leaf extract (OLE) and oleuropein on intracellular OS, cellular viability, apoptosis, necrosis and insulin secreting function in the INS-1 cell line.

RESEARCH DESIGN AND METHODS

Plant materials and extracts. To obtain oleuropein-rich fraction from olive leaves we used ethanol extraction of leaves previously extracted with petroleum ether. Petroleum ether was used for extraction of pigments and lipophylic material from olive leaves (free fatty/resin acids, sterols, waxes, steryl esters, triglycerides). Olive leaves were collected from the Balıkesir-Edremit region in Turkey in June 2008. Olive leaf samples were hand-picked randomly from the trees. They were dried in the shade and crushed. After crushing, 100 g of powdered leaves was extracted by stirring for 12 h at 50 °C in 500 mL petroleum ether. The petroleum extracts were filtered and dried. Then the residue was extracted by stirring 12 h at 50 °C with 500 mL of 95% ethanol. Following extraction, the ethanol phase was filtered and dried using mini spray dryer (Buchi Laboratory Equipment B-290, Switzerland) to obtain dry olive leaf extract (16.35 g). We used the European Pharmacopeia (EP 6th edn; vol 3, pp 2545–2546) method for quantitative analysis of oleuropein content in the ethanolic extract. We obtained 20.12 ± 0.18% oleuropein in olive leaf ethanolic extract.

Cell culture. INS-1 cells (kindly provided by Professor Claes Wollheim and Professor Pierre Maechler, University of Geneva, Switzerland) were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 1 mM Na-pyruvate, 55 μM 2-mercaptoethanol, 10 mM Hepes, pH 7.0–7.4. For assays the cells were detached by 10–15 min incubation with trypsin/EDTA. The cells were grown in a humidified incubator containing 5% CO_2 and 95% air at 37 °C.

Cytotoxicity assay (MTT). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium bromide (MTT) reduction assay was used as an indicator of cell damage and performed as previously described (Janjic & Wollheim, 1992). INS-1 cells were seeded in wells at a density of 3.0×10^4 cells per culture well. The cells were preincubated for 24 h with or without different concentrations of oleuropein (a range of doses between 10^{-1}–10^{-4} mmol/L) and olive leaf extract (10^{-1}–10^{-4} mg/mL), followed by incubation with H_2O_2 (0.035 mmol/L) in Krebs-Ringer buffer for 45 min at 37 °C. MTT was added to the final concentration of 0.5 mg/mL. After 2 h of MTT incubation, solubilization buffer (10% SDS in 0.01 mol/L HCl) was added and cells were gently resuspended. The absorbance at 570 nm was recorded with a microplate reader (Bio-Tek ELX800, BioTek Instruments Inc., Vermont, USA).

Acridine orange/Ethidium bromide (AO/EB) staining assay. Apoptotic and necrotic changes in the cells were determined morphologically by fluorescence microscopy with AO/EB staining as described by (Ribble *et al.*, 2005). INS-1 cells were seeded in 6-well plates at a density of 5.0×10^5 cells per culture well. Cells were preincubated for 24 h with or without oleuropein (0.1 mmol/L) or olive leaf extract (0.1 mg/mL) followed by incubation with H_2O_2 in Krebs-Ringer buffer for 45 min at 37 °C. Cells were collected and washed three times in phosphate buffered saline (PBS). Cells were centrifuged (1000 r.p.m.) and pellet was gently resuspended in 25 μL of medium containing 3 μL of dye mixture (100 μg/mL acridine orange and 100 μg/mL ethidium bromide in PBS). Ten microliters of cell-dye mixture was placed on a microscopic slide and images were collected using a fluorescence microscope (Leica Microsystems, Germany) with excitation at 488 nm and emission at 520 nm. For each well, three or more random pictures were obtained. Tests were done in triplicate and minimally 300 cells were counted for each slide.

Intracellular ROS generation. Intracellular ROS generation was evaluated with a modified method previously described by Rosenkranz *et al.* (1992). The cells were seeded in 96-well plate at a density of 15×10^5 cells/cm^2 and pre-incubated for 24 h with or without different concentrations of oleuropein (10^{-1}–10^{-4} mmol/L) or olive leaf extract (10^{-1}–10^{-4} mg/mL) followed by incubation with 15 μmol/L 2',7'-dichlorofluorescin diacetate (DCF-DA, Molecular Probes) in Krebs-Ringer buffer for 1 h at 37 °C. DCF-DA is a membrane permeable probe which is hydrolyzed by cellular esterases resulting in formation of non-fluorescent dichlorofluorescein (DCFH). When oxidized by free radicals, DCFH is converted to fluorescent DCF. After DCF-DA loading, the cells were incubated with 0.035 mmol/L H_2O_2 for 45 min in Krebs-Ringer buffer at 37 °C. The fluorescence of DCF was quantified at an excitation wavelength of 485 nm and emission wavelength of 528 nm in a microplate fluorometer (Modulus microplate multimode reader, Turner Bio Systems, USA).

Insulin secretion. INS-1 cells were seeded in 24-well plates at a density of 5.0×10^5 (in 1 mL medium) cells per culture well. Cells were preincubated for 24 h with or without oleuropein (0.1 mmol/L) or olive leaf extract (0.1 mg/mL) followed by incubation with H_2O_2 (0.035 mmol/L) in Krebs-Ringer buffer for 45 min at 37 °C. The cells were then washed and preincubated for 30 min in glucose-free buffer supplemented with 0.1% BSA (bovine serum albumin) and containing (in mmol/L) NaCl 125, KCl 5.9, $MgCl_2$ 1.2, $CaCl_2$ 1.3, Hepes 25 titrated to pH 7.4 with NaOH. The glucose-free buffer was replaced by the same buffer supplemented with 16.7 mM glucose and the cells were incubated for 120 min at 37 °C. The cells were sedimented by centrifugation for 5 min at 2000 r.p.m., and then the supernatants were analyzed for insulin content with an enzyme-linked immunosorbent assay (ELISA) kit (Mercodia, Uppsala, Sweden).

Glutathione peroxidase and catalase activity assay. Glutathione peroxidase (GPx) activity and catalase activity were assayed by using colorimetric assay kits (all from Cayman Chemical Ann Arbor, MI, USA). Cells 2.0×10^6 in 6-well plates for each experiment, were preincubated for 24 h with or without oleuropein (0.1 mmol/L) or olive leaf extract (0.1 mg/mL) followed by incubation with H_2O_2 (0.035 mmol/L) in Krebs-Ringer buffer for 45 min at 37 °C. The cells were scraped and homogenized according to the kit protocols. Absorbance at a specific wavelength for each assay was recorded with a microplate reader.

Chemicals. All cell culture products were obtained from GIBCO® Invitrogen, USA. H_2O_2 was purchased

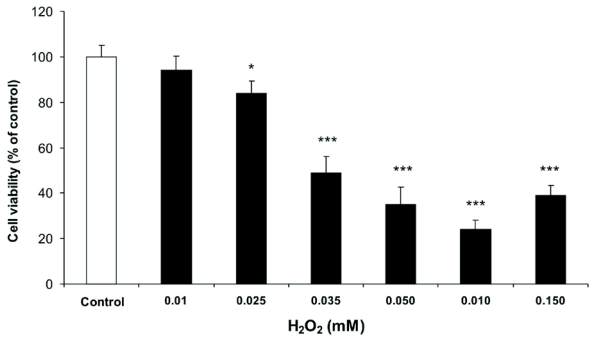

Figure 1. Cytotoxicity of H_2O_2 assessed by MTT viability test in INS-1 cells
*$P<0.05$, ***$P<0.001$ *vs* control.

from Merck (Merck & Co., Inc. USA). Oleuropein and all other chemicals, unless otherwise stated, were from Sigma (Sigma-Aldrich Co., USA).

Statistics. Each experiment was performed three times. Data are expressed as mean ± S.D. One-way analysis of variance (ANOVA) was used in multiple group comparisons. Difference with $P<0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

Pancreatic β-cells are essential for glucose homoeostasis. The dysfunction of these insulin-secreting cells results in diabetes development. Hydrogen peroxide (H_2O_2) has been demonstrated to be a critical factor in physiological cellular signal transduction for glucose-stimulated insulin secretion in β-cells (Pi *et al.*, 2007). However, excessive and/or sustained H_2O_2 production can directly or indirectly disturb the integrity and the function of these cells. Such disturbances in β-cells contribute to the pathogenesis of diabetes (Del Guerra *et al.*, 2005; Kaneto *et al.*, 2007).

H_2O_2 is used extensively in cell culture studies for induction of OS instead of the known insulinoma cell specific toxins like alloxan and streptozotocin (Maechler *et al.*, 1999; Pi *et al.*, 2007; Kaneto *et al.*, 2007; Hou *et al.*, 2008). The exposure to H_2O_2 produces injury in islets *via* suppressing hyperpolarization of the mitochondrial membrane and it impairs glucose-stimulated insulin secretion in INS-1 cells (Maechler *et al.*, 1999). In our study, starting with the concentration of 25 μmol/L H_2O_2, a significant drop in INS-1 cell viability as assessed by MTT (89.1 ± 8.8% of control) was obtained after 45 min period of treatment (Fig. 1). The concentration of 35 μmol/L H_2O_2, which decreases the viability of the cells by about 50% was used for further experiments (Fig. 1). Oleuropein and OLE were non-toxic at concentrations up to 0.1 mmol/L and 0.1 mg/mL, respectively, as assessed by reduction of MTT (not shown). A 24-hour preincubation with the OLE or oleuropein led to a significant improvement in the viability of H_2O_2-exposed INS-1 cells, but a complete amelioration was not obtained (Fig. 2). Using AO/EB double staining, four cell stages were identified after exposure to H_2O_2-induced stress: living cells uniformly green stained with non-fragmented chromatin, cells with bright green and orange nuclei and condensed or fragmented chromatin (early and late apoptotic cells, respectively), and cells with red intact nuclei (necrotic cells) (Fig. 3). Exposure to H_2O_2 significantly increased the proportion of apoptotic and necrotic cells (Fig. 4). Preincubation with oleuropein or OLE partly, but significantly increased the proportion of living cells and prevented cell death by necrosis (Fig. 4). Interestingly, neither OLE nor oleuropein did significantly affect the proportion of apoptotic cells after H_2O_2 exposure (Fig. 4). As expected, increased intracellular ROS production (308.1 ± 56.7% of control) was found in H_2O_2-treated cells (Fig. 5). Although oleuropein and OLE were able partly to decrease ROS production, these effects were only seen at the highest concentrations of these antioxidants (Fig. 5). Our data confirm the ability of OLE to control ROS generation and/or scavenge the intracellular ROS reported in early studies (Visioli *et al.*, 1998; Oliveras-López *et al.*, 2008). Visioli and coworkers (1998) reported the ROS-neutralizing property of oleuropein by both in an *in vitro* xanthine/xanthine oxidase system and in an *ex vivo* human polymorphonuclear neutrophil model of superoxide formation.

In our study, the H_2O_2-induced OS caused a decrease in insulin secretion to 44.1 ± 6.1% of that in control cells (Fig. 6A). These data are in agreement with those of Xiong *et al.* (2006) who reported that 2-hour incubation with H_2O_2 caused a decrease of glucose-induced insulin secretion in isolated pancreatic islets. When β-cells were exposed to OS inducers, insulin gene expression was shown to be decreased by the activation of the JNK pathway (Tanaka *et al.,* 2002; Sakai *et al.*, 2003; Robertson *et al.*, 2007). The preserving effects of various natural antioxidants on insulin content and insulin mRNA of β-cells have already been demonstrated by others (Kaneto *et al.*, 2007). It is well know that olive polyphenols are powerful antioxidants *in vitro* and possess other *in vivo* biological activities that could account for their beneficial health effects (Covas, 2007). Interestingly, in H_2O_2-treated INS-1 cells, the preincubation with oleuropein completely restored insulin secretion to control levels, but the effect of OLE, composed of antioxidant mixture, was only partial (Fig. 6A).

To prevent ROS-induced destruction, cells protect their vital functions by enzymatic or non-enzymatic mechanisms. These include a number of enzymes such as glutathione peroxidase (GPx) which catalyze reactions to get rid of the oxidative effects of ROS. H_2O_2 is converted into water and molecular oxygen by GPx and catalase. In the present study, while GPx activity was significantly inhibited, catalase activity was increased after H_2O_2 addition to INS-1 cell (Fig. 6B and 6C, respec-

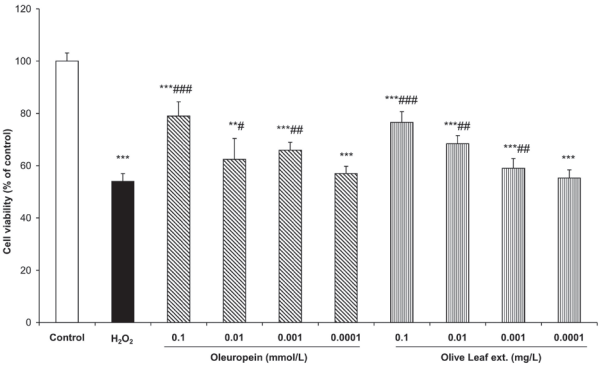

Figure 2. Effects of oleuropein and olive leaf extract on cell viability of H_2O_2–treated INS-1 cells
*$P<0.05$, **$P<0.01$, ***$P<0.001$ *vs* control; #$P<0.05$, ##$P<0.01$, ###$P<0.001$ *vs* H_2O_2.

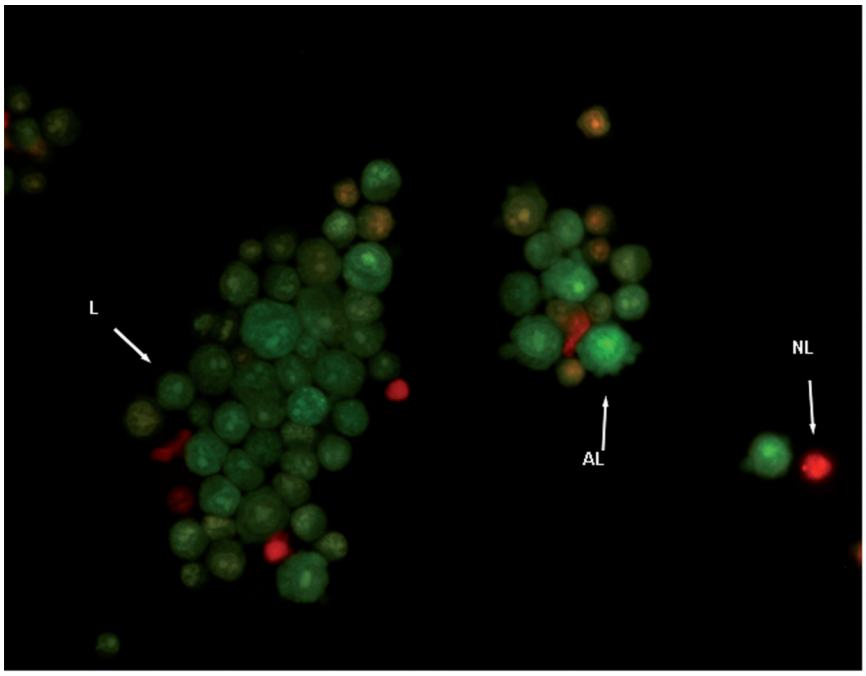

Figure 3. AO/EB staining of INS-1E cells treated with H_2O_2
(L) Living cell, **(AL)** Apoptotic cell, **(NL)** Necrotic cell. A 40× objective was used.

tively). Interestingly, OLE afforded a complete preservation of the GPx activity, but oleuropein had no significant effect against the H_2O_2-induced depression (Fig. 6B and 6C, respectively). The preservation of GPx activity allowed the normal content of GSH to be maintained. Previously, the intensity of insulin secretion has been found to closely correlate with the activity of GPx as demonstrated by using cysteine or synthetic antioxidants against H_2O_2-induced toxicity in cell culture (Xiong *et al.*, 2006; Oliveras-López *et al.*, 2008). Our experiments demonstrated that H_2O_2 activates catalase as a physiological defense response against oxidative attack (Fig. 6C). Interestingly, OLE and oleuropein treatments enhanced this increase even further (Fig. 6C). The exacerbating action of OLE and oleuropein on catalase in the presence of H_2O_2 is likely related to their pro-oxidant properties. A dual action of polyphenols as both antioxidant and pro-oxidant, has been demonstrated in cell culture systems with the common plant polyphenols, quercetin and epigallocatechin-3-gallate (Raza & John, 2005; Robaszkiewicz *et al.,* 2007). These polyphenols have been shown to decrease production of ROS in the cells but also trigger the production of peroxides in the medium in the presence of H_2O_2 (Raza & John, 2005; Robaszkiewicz *et al.,* 2007). Thus, the effects of OLE polyphenols on redox systems in our model seem to be complex and might include both antioxidant effects and induction of OS due to formation of ROS in the extracellular medium. Regardless of the localization of catalase, its increased activity reflects an increased catalase-dependent pathway to consume H_2O_2 and other peroxides. While catalase is primarily located in peroxisomes, it has been reported that extracellular peroxidase activity of catalase is critical for ensuring efficient protection against exogenous H_2O_2 (Hervé-Grépinet *et al.,* 2008). Generally, oleuropein is believed to be a key active ingredient responsible for the benefits of OLE, and the anti-diabetic properties of olive leaves were until recently attributed only to oleuropein. Oleuropein in olive leaf ethanolic extract obtained in this study was combined with other phytochemicals that naturally occur in live leaves, including other phenolic compounds and flavones such

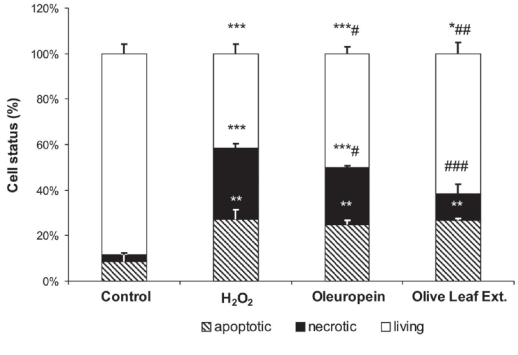

Figure 4. Effects of oleuropein and olive leaf extract on H_2O_2-induced INS-1 cell death
H_2O_2 was used at 0.035 mmol/L, oleuropein at 0.1 mmol/L and olive leaf extract at 0.1 mg/mL. Apoptotic (▨), necrotic (■) and living (□) cells were counted following AO/EB staining. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs* H_2O_2.

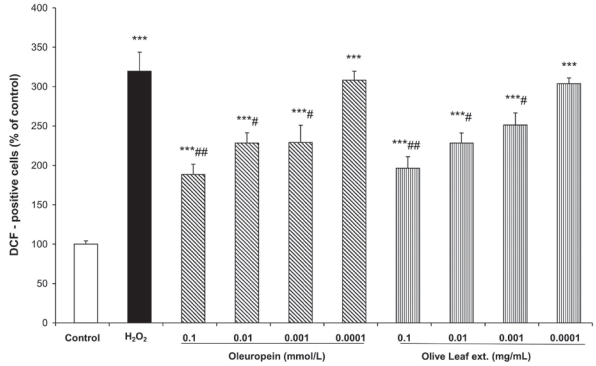

Figure 5. Effects of oleuropein and olive leaf extract on intracellular ROS generation after exposure of INS-1 cells to H_2O_2 (0.035 mmol/L)
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$ *vs* H_2O_2.

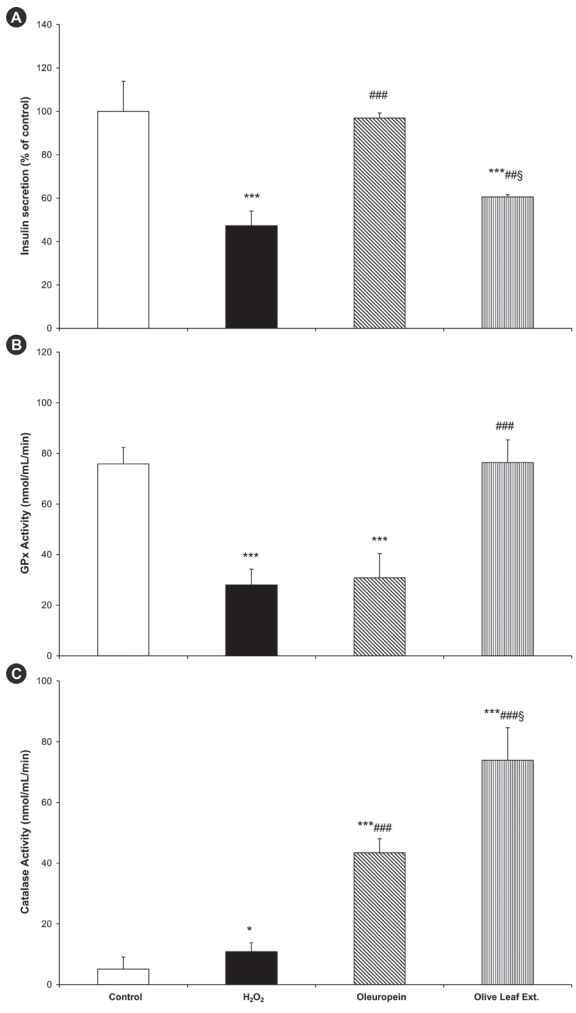

Figure 6. Effects of oleuropein and olive leaf extract on insulin secretion (A), glutathione peroxidase (GPx) activity (B) and catalase activity (C) after exposure of INS-1 cells to H_2O_2 (0.035 mmol/L)
$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ *vs* control; $^{\#}P<0.05$; $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ *vs* H_2O_2 ; $^{\S}P<0.001$ *vs* oleuropein.

as tyrosol and hydroxytyrosol (Gordon *et al*., 2001). The literature indicates that most of the compounds present in olive leaves, have antioxidant properties, preventing or reducing the deleterious effects of OS associated with diabetes (Eidi & Darzi, 2009; Hamden *et al.,* 2009; Cvjetićanin *et al.,* 2010); however, their effects on insulin secretion in experimental cell culture models have not been studied to date. Purified hydroxytyrosol, the major phenolic compound from olive mill waste, has shown hypoglycemic and antioxidant effects in rats (Hamden *et al.,* 2009). Others also isolated and identified another constituent, triterpene oleanolic acid, from olive leaves. It lowered serum glucose and insulin levels in mice fed a high fat diet and enhanced glucose tolerance (Sato *et al.,* 2007). In the case of the present work, it is difficult to explain why oleuropein alone was found to be more effective in normalizing insulin secretion by INS-1 cell than was the antioxidant mixture of OLE. This finding implies different effects of the active ingredients of OLE on the redox-sensitive regulation of insulin secretion. The positive regulatory effect of oleuropein in OLE might be overshadowed by properties of the other constituents of the OLE mixture. It has been demonstrated that the basal physiological effect of H_2O_2 on glucose-stimulated insulin secretion disappears at high H_2O_2 concentrations, when the antioxidative defense is activated (Pi *et al.,* 2007), as was the case of our model using H_2O_2 plus OLE. The benefits of olive leaf compounds are supported by recent literature data reporting olive leaf-derived polyphenols as therapeutic agents delaying the progression of advanced glycation end products-mediated inflammatory diseases such as diabetes (Chandler *et al.,* 2010).

Acknowledgement

Financial support by COST B35 (Lipid Peroxidation Associated Disorders: LPO) grants, the Scientific and Technical Research Council of Turkey (TÜBİTAK) (Project no. 106S025, SBAG-3303) and Gazi University-Ankara is gratefully acknowledged.

REFERENCES

Chandler D, Woldu A, Rahmadi A, Shanmugam K, Steiner N, Wright E, Benavente-García O, Schulz O, Castillo J, Münch G (2010) Effects of plant-derived polyphenols on TNF-alpha and nitric oxide production induced by advanced glycation endproducts. *Mol Nutr Food Res* **54** (Suppl 2)**:** S141–S150.

Cnop M, Welsh N, Jonas JC, Jörns A, Lenzen S, Eizirik DL (2005) Mechanisms of pancreatic beta-cell death in type 1 and type 2 diabetes**:** many differences, few similarities. *Diabetes* **54** (Suppl 2)**:** S97–S107.

Covas MI (2007) Olive oil and the cardiovascular system. *Pharmacol Res* **55:** 175–186.

Cvjetićanin T, Miljković D, Stojanović I, Dekanski D, Stosić-Grujicić S (2010) Dried leaf extract of *Olea europaea* ameliorates islet-directed autoimmunity in mice. *Br J Nutr* **103:** 1413–1424.

Del Guerra S, Lupi R, Marselli L, Masini M, Bugliani M (2005) Functional and molecular defects of pancreatic islets in human type 2 diabetes. *Diabetes* **54:** 727–735.

Eidi A, Eidi M, Darzi R (2009) Antidiabetic effect of *Olea europaea* L. in normal and diabetic rats. *Phytother Res* **23:** 347–350.

Eizirik DL, Mandrup-Poulsen TA (2001) A choice of death — the signal-transduction of immune-mediated β-cell apoptosis. *Diabetologia* **44:** 2115–2133.

Gariboldi P, Jommi G, Verotta L (1986) Secoiridoids from *Olea europaea*. *Phytochemistry* **25:** 865–869.

Gembal M, Gilon P, Henquin JC (1992) Evidence that glucose can control insulin secretion independently from its action on ATP-sensitive K+ channels in mouse B cells. *J Clin Invest* **89:** 1288–1295.

Gonzalez M, Zarzuelo A, Gamez MJ, Utrilla MP, Jimenez J, Osuna I (1992) Hypoglycemic activity of olive leaf. *Planta Medica* **58:** 513–515.

Gordon MH, Paiva-Martins F, Almeida M (2001) Antioxidant activity of hydroxytyrosol acetate compared with that of other olive oil polyphenols. *J Agric Food Chem* **49:** 2480–2485.

Güneş A, Ceylan A, Sarioglu Y, Stefek M, Bauer V, Karasu C (2005) Antioxidants in Diabetes-induced Complications (ADIC) Study Group. Reactive oxygen species mediate abnormal contractile response to sympathetic nerve stimulation and noradrenaline in the vas deferens of chronically diabetic rats: effects of *in vivo* treatment with antioxidants. *Fundam Clin Pharmacol* **19:** 73–79.

Hamden K, Allouche N, Damak M, Elfeki A (2009) Hypoglycemic and antioxidant effects of phenolic extracts and purified hydroxytyrosol from olive mill waste *in vitro* and in rats. *Chem Biol Interact* **180:** 421–432.

Heimler D, Pieroni A, Tattini M, Cimato A (1992) Determination of flavonoids, flavonoid glycosides and biflavonoids in *Olea europaea* L. leaves. *Chromatographia* **33:** 369–373.

Hervé-Grépinet V, Veillard F, Godat E, Heuzé-Vourc'h N, Lecaille F, Lalmanach G (2008) Extracellular catalase activity protects cysteine cathepsins from inactivation by hydrogen peroxide. *FEBS Lett* **582:** 1307–1312.

Hou ZQ, Li HL, Zhao JJ, Li GW (2008) Impairment of pancreatic islet beta cell function induced by intermittent high glucose through oxidative and endoplasmic reticulum stress: experiment with rat pancreatic islet beta cells. *Zhonghua Yi Xue Za Zhi* **88:** 2002–2004.

Ihara Y, Toyokuni S, Uchida K, Odaka H, Tanaka T, Ikeda H, Hiai H, Seino Y, Yamada Y (1999) Hyperglycemia causes oxidative stress in pancreatic beta-cells of GK rats, a model of type 2 diabetes. *Diabetes* **48:** 927–932.

Janjic D, Wollheim CB (1992) Islet cell metabolism is reflected by the MTT (tetrazolium) colorimetric assay. *Diabetologia* **35:** 482–485.

Jemai H, El Feki A, Sayadi S (2009) Antidiabetic and antioxidant effects of hydroxytyrosol and oleuropein from olive leaves in alloxan-diabetic rats. *J Agric Food Chem* **57:** 8798–8804.

Kaneto H, Matsuoka TA, Katakami N, Kawamori D, Miyatsuka T, Yoshiuchi K, Yasuda T, Sakamoto K, Yamasaki Y, Matsuhisa M (2007) Oxidative stress and the JNK pathway are involved in the development of type 1 and type 2 diabetes. *Curr Mol Med* **7:** 674–686.

Latha M, Pari L, Sitasawad S, Bhonde R (2004) *Scoparia dulcis*, a traditional antidiabetic plant, protects against streptozotocin induced oxidative stress and apoptosis *in vitro* and *in vivo*. *J Biochem Mol Toxicol* **18:** 261–272.

Lin E, Wang Y, Mehendale S, Sun S, Wang CZ, Xie JT, Aung HH, Yuan CS (2008) Antioxidant protection by American ginseng in pancreatic beta-cells. *Am J Chin Med* **36:** 981–988.

Maechler P, Jornot L, Wollheim CB (1999) Hydrogen peroxide alters mitochondrial activation and insulin secretion in pancreatic beta cells. *J Biol Chem* **274:** 27905–27913.

Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P (2004) Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* **145:** 667–678.

Oliveras-López MJ, Berná G, Carneiro EM, López-García de la Serrana H, Martín F, López MC (2008) An extra-virgin olive oil rich in polyphenolic compounds has antioxidant effects in OF1 mice. *J Nutr* **138:** 1074–1078.

Pi J, Bai Y, Zhang Q, Wong V, Floering LM, Daniel K, Reece JM, Deeney JT, Andersen ME, Corkey BE, Collins S (2007) Reactive oxygen species as a signal in glucose-stimulated insulin secretion. *Diabetes* **56:** 1783–1791.

Raza H, John A (2005) Green tea polyphenol epigallocatechin-3-gallate differentially modulates oxidative stress in PC12 cell compartments. *Toxicol Appl Pharmacol* **207:** 212–220.

Rhodes CJ (2005) Type 2 diabetes-a matter of beta-cell life and death? *Science* **307:** 380–384.

Ribble D, Goldstein NB, Norris DA, Shellman YG (2005) A simple technique for quantifying apoptosis in 96-well plates. *BMC Biotechnol* **5:** 12.

Robaszkiewicz A, Balcerczyk A, Bartosz G (2007) Antioxidative and prooxidative effects of quercetin on A549 cells. *Cell Biol Int* **31:** 1245–1250.

Robertson R, Zhou H, Zhang T, Harmon JS (2007) Chronic oxidative stress as a mechanism for glucose toxicity of the beta cell in type 2 diabetes. *Cell Biochem Biophys* **48:** 139–146.

Robertson RP, Harmon J, Tran PO, Tanaka Y, Takahashi H (2003) Glucose toxicity in beta-cells: type 2 diabetes, good radicals gone bad, and the glutathione connection. *Diabetes* **52:** 581–587.

Robertson RP, Harmon JS (2006) Diabetes, glucose toxicity, and oxidative stress: A case of double jeopardy for the pancreatic islet beta cell. *Free Radic Biol Med* **41:** 177–184.

Romani A, Baldi A, Tattini M, Vincieri FF (1994) Extraction, purification procedures and HPLC-RI analysis of carbohydrates in olive (*Olea europaea* L.) plants. *Chromatographia* **39:** 35–39.

Rosenkranz AR, Schmaldienst S, Stuhlmeier KM, Chen W, Knapp W, Zlabinger GJ (1992) Microplate assay for the detection of oxidative products using 2',7'-dichlorofluorescein-diacetate. *J Immunol Methods* **156:** 39–45.

Sakai K, Matsumoto K, Nishikawa T, Suefuji M, Nakamaru K, Hirashima Y, Kawashima J, Shirotani T, Ichinose K, Brownlee M, Araki E (2003) Mitochondrial reactive oxygen species reduce insulin secretion by pancreatic beta-cells. *Biochem Biophys Res Commun* **300:** 216–222.

Sato H, Genet C, Strehle A, Thomas C, Lobstein A, Wagner A, Mioskowski C, Auwerx J, Saladin R (2007) Anti-hyperglycemic activity of a TGR5 agonist isolated from *Olea europaea*. *Biochem Biophys Res Commun* **362:** 793–798.

Soni MG, Burdock GA, Christian MS, Bitler CM, Crea R (2006) Safety assessment of aqueous olive pulp extract as an antioxidant or antimicrobial agent in foods. *Food Chem Toxicol* **44:** 903–915.

Speroni E, Guerra MC, Minghetti A Crespi-Perellino N, Pasini P, Piazza F (1998) Oleuropein evaluated *in vitro* and *in vivo* as an antioxidant. *Phytother Res* **12:** 98–100.

Tanaka Y, Tran PO, Harmon J, Robertson RP (2002) A role for glutathione peroxidase in protecting pancreatic beta cells against oxidative stress in a model of glucose toxicity. *Proc Natl Acad Sci USA* **99:** 12363–12368.

Visioli F, Bellomo G, Galli C (1998) Free radical scavenging properties of olive oil polyphenols. *Biochem Biophys Res Commun* **247:** 60–64.

Xiong FL, Sun XH, Gan L, Yang XL, Xu HB (2006) Puerarin protects rat pancreatic islets from damage by hydrogen peroxide. *Eur J Pharmacol* **529:** 1–7.

Yülek F, Or M, Ozoğul C, Isik AC, Ari N, Stefek M, Bauer V, Karasu C (2007) Effects of stobadine and vitamin E in diabetes-induced retinal abnormalities: involvement of oxidative stress. *Arch Med Res* **38:** 503–511.