Quercetin and hydroxytyrosol attenuates xanthine/xanthine oxidase-induced toxicity in H9c2 cardiomyocytes by regulation of oxidative stress and stress-sensitive signaling pathways

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Abstract. The increased activity of xanthine/xanthine oxidase (X/XO) has been suggested as a risk factor for heart disease and herbal polyphenols exhibits cardioprotection *in vitro* and *in vivo*. To understand the cardioprotective action mechanisms of polyphenol quercetin and hydroxytyrosol, the expression levels of stress-responsive proteins were studied in X/XO-induced toxicity model of H9c2 cardiomyocyocytes. Pretreatment with each polypenol (0.1–10 μg/ml; 24 h) enhanced viability ($p < 0.01$; MTT test) and inhibited reactive oxygen species (ROS) generation ($p < 0.001$; H2DCFDA assay) against 12 h exposure to a free radical generating system, X (0.5 mM) and XO (5 mU/ml). Western blotting experiments showed that X/XO increases the phosphorylation of downstream substrate of p38, MAPK-activated protein kinase 2 (MAPKAPK-2), p44/42-MAPK (Erk1/2) and cleaved caspase-3 ($p < 0.001$, *vs.* Control), however inhibits the levels of phosphorylated c-Jun and Hsp27 ($p < 0.01$, *vs.* Control). Pretreatment with quercetin or hydroxytyrosol attenuated the phosphorylation of MAPKAPK-2 and cleaved caspase-3 in X/XO-exposed cells ($p < 0.01$, *vs.* X/XO). Hydroxytyrosol enhanced the reduction of phosphorylation of a transcriptional target c-Jun and led to overphosphorylation in protective proteins, p44/42-MAPK and Hsp27 in X/XO-exposed cells ($p < 0.01$, *vs.* X/XO). Our data suggest that quercetin and hydroxytyrosol protects cardiomyocytes against X/XO-induced oxidative toxicity by diminishing intracellular ROS and the regulation of stress-sensitive protein kinase cascades and transcription factors.

Key words: Quercetin — Hydroxytyrosol — H9c2 cardiomyocyte — Xanthine/xanthine oxidase — Stress-sensitive signaling

Introduction

Oxidative stress and reactive oxygen species (ROS) have been widely implicated in endothelial damage, progression of atherosclerosis, dilated cardiomyopathy and in ischemia/reperfusion injury (Karasu 2010). Polyphenols have been gaining recognition as strategies to reduce ROS and acceptance as potential therapeutic agents to protect individuals from cardiovascular diseases (Khurana et al. 2013). Although the beneficial effects of polyphenols have been attributed primarily to their antioxidant capacity and their ability to modulate cellular antioxidant defense, the recent studies provided evidence of polyphenols as modulators of cellular signaling pathways (Incani et al. 2010). In this regards, for describe how polyphenols counteract intracellular ROS as well as modulate signaling pathways to enhance health outcomes we investigated the protective effects of olive leaf polyphenolic compounds against oxidative toxicity and apoptotic death signaling induced by hydrogen peroxide (H_2O_2), cytokines or 4-HNE in cardiomyocytes (Bali et al. 2014) and insulin releasing cells (Cumaoğlu et al. 2011a, 2011b). One of the major polyphenols present in the leaves of olive (*Olea europaea* L.), hydroxytyrosol, shows

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a variety of pharmacological activities such as antioxidant, anti-inflammatory, anticancer, kidney-protective and neuroprotective properties, and a beneficial effect on the cardiac system (Hu et al. 2014). Hydroxytyrosol, a metabolite of oleuropein, exhibits protection against cardiac risk through the down-regulation of adhesion molecules, inhibiting platelet activity and improving vascular stability (Rafehi et al. 2012). In addition to hydroxytyrosol, quercetin, another polyphenol belonging to the flavonoid group is found in the fruit and the leaves of olive, has been suggested to be of benefit in ameliorating cardiovascular health *via* eNOS upregulation, the reduction of oxidative stress, increasing the activities of antioxidant enzymes and anti-clotting and anti-proliferative abilities (Chen et al. 2013; Ergin et al. 2013; Miles et al. 2014).

On the other hand, hypoxia-reoxygenation of the heart results in the activation of xanthine oxidase, leading to increase production of superoxide anion and hydroxyl (Durot et al. 2000). The superoxide anion generating xanthine/xanthine oxidase (X/XO) system has been shown to induce cardiomyocyte death, which is a hallmark of ischemic heart disease (Durot et al. 2000; Miao et al. 2013). However, the role of quercetin and hydroxytyrosol and underlying cellular mechanism in cardioprotection against X/XO has not been clearly elucidated with respect to the stress-activated transcription factors and signaling pathways. Thus, the present study was carried out to investigate the capacity of quercetin and hydroxytyrosol to modulate cell viability and ROS production in a superoxide anion-induced stress model of H9c2 cells *via* transaction to X/XO. We also evaluated the effects of quercetin and hydroxytyrosol on survival or death signaling molecules, phospho-MAPKAPK-2, phospho-p44/42-MAPK, cleaved caspase-3, phospho-c-Jun and phospho-Hsp27, in the presence of X/XO.

Material and Methods

Cell culture

The embryonic rat heart-derived myogenic cell line H9c2 was purchased from the American Tissue Type Collection (#CRL-1446). The H9c2 cells were cultured in 75-cm^2 TC flasks in DMEM supplemented with 10% FBS, 2 mM L-glutamine, and 1% penicillin-streptomycin. The cells were cultured at 37°C in a humidified atmosphere containing 95% air/5% CO_2. Harvested cells were plated in 96-well microplates (1×10^4 cells/well) and allowed to grow to confluence over 24 hours before use. Cells were incubated with or without quercetin or hydroxytyrosol (0.1–10 μg/ml) for 24 h. These effective concentrations and the incubation time were obtained in preliminary studies according to the literature (Bali et al. 2014).

Induction of oxidative stress

Oxidative stress was induced by incubating H9c2 cells with xanthine oxidase (XO) (50 mU/ml) in the presence of its substrate xanthine (X) (1 mM) for 12 h. Exposure of cardiomyocytes in culture to X/XO causes significant cell injury and this has been demonstrated to be due to generation of ROS and in particular of superoxide as well as OH produced from H_2O_2 by the iron-catalysed Fenton reaction (Du et al. 2007; Miao et al. 2013).

Evaluation of cell viability

Cell viability was measured using an MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay, based on the MTT conversion into formazan crystals using mitochondrial dehydrogenases (Janjic and Wollheim 1992). Briefly, H9c2 cells were plated at a density of 2×10^4 cells/well in 96-well plates. After the treatment, the culture medium was replaced with 200 μl of MTT solution, that is, 5 mg/ml stock solution in phosphate buffered saline (PBS), diluted with culture medium to the final concentration of 0.5 mg/ml. After a 4-hour incubation at 37°C, this solution was removed and the formazan was solubilized in 100 μl of dimethyl sulfoxide. The absorbance was monitored at 572 nm using an automated microplate reader (PowerWave XS2; BioTek Instruments Inc., Winooski, VT, USA). Cell viabilities were calculated by comparing results to control cells considered 100% viable. The cells were assayed in quadruplicate, and at least two independent experiments were carried out.

Measurement of ROS in cells

The oxidation of 2',7'-dichlorofluorescin (H_2DCF) to 2',7'-dichlorofluorescein (DCF) was used for the quantitation of intracellular ROS production. Cells were seeded on 96-well plates at 2×10^4 cells. The cells pretreated with each phenolic compounds in concentrations of 0.1 and 10 μM for 24 h and then exposed to X (0.5 mM) and XO (5 mU/ml) for 12 h. Cell culture plates were washed twice with Hank's balanced saline solution and then incubated with 10 μM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) for one hour at 37°C in the dark. Oxidation of the dye by intracellular ROS generates a fluorescent 2',7'-dichlorofluorescin (DCF) signal. Fluorescence was measured using the excitation/emission wavelengths 485/530 nm with a Biotek FL600 microplate reader (BioTek Instruments Inc., Winooski, VT, USA) (Rosenkranz et al. 1992).

Western blotting

H9c2 cells cultured in 60-mm petri dishes (Sarstedt) were lysedin 250 μl of lysis buffer (1% Triton, 20 mM Tris-HCl

(pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA, 1 mM EGTA, 2.5 mM sodium pyrophosphate, 1 mM beta-glycerophosphate, 1 mM Na_3VO_4, and 1 μg/ml leupeptin) supplemented with 1 mM PMSF (Roche Diagnostics, Istanbul, Turkey). Protein concentrations were determined by using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific Inc., IL, USA). Protein lysates (20 μg) were heated for 5 min at 94°C in Laemmli sample buffer containing 5% β-mercaptoethanol and loaded on 4–15% Tris-glycine SDS-PAGE gels, and then transferred electrophoretically to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk for 1 h and incubated overnight at 4°C with phospho-MAPKAPK-2 (Thr334), phospho-Hsp27 (Ser82), phospho-p44/42 (MAPK (Thr 202/Tyr204), phospho-c-Jun (Ser73), and cleaved caspase-3 (Asp175) antibodies (Cell Signaling Technology-SACEM, Ankara, Turkey). Protein bands were detected with horseradish peroxidase-conjugated anti-rabbit secondary antibodies (Cell Signaling Technology-SACEM, Ankara, Turkey) and visualized by West-Femto ECL reagents (Thermo Fisher Scientific Inc., IL, USA). Chemiluminescent signals of immunoblots were documented by using Gel Logic 2200 Pro (Carestream Health). The intensity of specific proteins was quantified by using Carestream Molecular Image Software and ImageJ Software (v. 1.4; National Institutes of Health). GAPDH were used as internal control for the Western blot analysis.

Materials

Hydroxytyrosol (HPLC grade > 98%; Applichem A5303 Lot# 11120406) was purchased from Applichem (Darmstadt, Germany). Quercetin was from Sigma-Aldrich (HPLC grade ≥ 98% Q4951 Lot# 060M1196 V). Fetal bovine serum (FBS) was acquired from Sigma-Aldrich Co. (LLC, St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Thermo Scientific (Waltham, MA, USA). Penicillin and streptomycin were obtained from Lonza (Walkersville, MD, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was supplied from (Merck, Co., Darmstadt, Germany). Dimethyl sulfoxide (DMSO), 2',7'-dichlorodihydrofluorescein diacetate (H_2D-CFDA) dye and Hank's balanced saline solution were purchased from Fisher Scientific Inc. (Waltham, MA, USA).

Statistical analysis

Experiments were performed at least three times (more than three times for MTT and H_2DCFDA experiments) with the data presented as mean values ± SD. Means and standard deviations were calculated with SPSS 20.0 software (Chicago, USA). One-way ANOVA along with the post-hoc Tukey's test was employed for statistical analysis. To verify the hypothesis about the equality of variances, Levene's test was employed. Results were considered statistically significant for $p < 0.05$.

Results

In present study, the stimulation of H9c2 cells with X (0.5 mM) and XO (5 mU/ml) evoked ROS generation ($p < 0.001$; H_2DCFDA assay) and decreased the viability of cells ($p < 0.001$; MTT assay) as shown in Figure 1. The preincubation with quercetin or hydroxytyrosol (0.1–10 μg/ml, 24 h) completely inhibited the X/XO-induced generation of intracellular ROS ($p < 0.001$) when compared to X/XO (0.5 mM and 5 mU/ml) exposed group. Neither quercetin nor hydroxytyrosol completely improved the viability of cells against X/XO-induced toxicity; however, the effect of each polyphenol was significant and concentration-dependent. In comparison with untreated cells, the viability increased significantly when the cells preincubated with quercetin or hydroxytyrosol at the concentrations of 0.1 μg/ml ($p < 0.01$) and 10 μg/ml ($p < 0.001$).
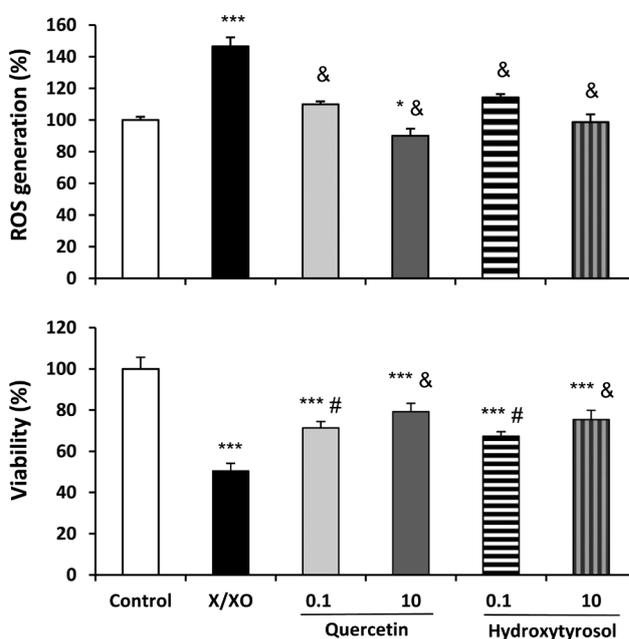
Figure 1. Effect of quercetin and hydroxytyrosol on intracellular ROS generation (H_2DCFDA assay) and the viability (MTT assay) of H9c2 cells exposed to xanthine/xanthine oxidase (X/XO). The cells were pre-incubated with quercetin or hydroxytyrosol (0.1 and 10 μg/ml) for 24 h and then exposed to X/XO (0.5 mM/5 mU/ml) for 12 h. All data are expressed as the means ± SD (ANOVA). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs.* Control; # $p < 0.01$, & $p < 0.001$ *vs.* X/XO.
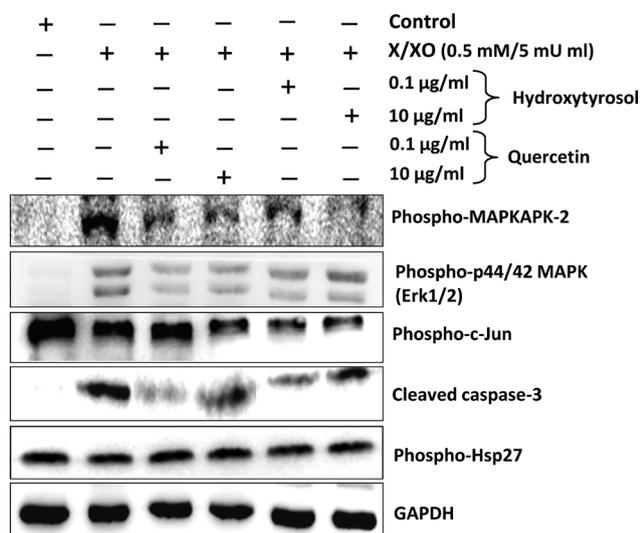
In this study, Western blotting analysis showed that cardiomyocyte levels of phosphorylated MAPKAPK-2, an index of p38 MAP kinase activity were significantly elevated in X/XO-exposed cells (Fig. 2 and Fig. 3). However, especially quercetin as well as hydroxytyrosol significantly inhibited superoxide-induced MAPKAPK-2 phosphorylation in X/XO-exposed cells. X/XO exposure increased phosphorylation of p44/42 MAPK (Erk1/2) (Thr202/Tyr204) in H9c2 cells compared to control cells ($p < 0.001$) (Fig. 2 and Fig. 3). Especially quercetin, at low concentration, inhibited Erk1/2 against to X/XO induction. However, Erk2 was slightly increased at the higher concentrations of both polyphenolic compounds. On the other hand, we demonstrated that X/XO exposure decreased phosphorylation of Hsp27 ($p < 0.01$). While quercetin significantly and concentration-dependently reversed X/XO-induced down-regulation of p-Hsp27, hydroxytyrosol led to further increase in the phosphorylation of Hsp27. We further found that the levels of phosphorylated (active) c-Jun was significantly reduced in X/XO exposed cells compared to control group ($p < 0.01$). Preincubation with quercetin reversed p-c-Jun down regulation, but hydroxytyrosol inhibited p-c-Jun in X/XO exposed cells more than in the cells exposed of X/XO alone (Fig. 2 and Fig. 3). Furthermore, X/XO exposure led to an increase


Figure 2. Western blot analysis of cell lysates before (Control) or after X/XO (0.5 mM/5 mU/ml) exposure in untreated (-) or pretreated (+) cells with quercetin and hydroxytyrosol are demonstrating myocardial level of survival or death proteins. Cell lysates (30 μg) from cells treated as indicated in the figure were analyzed by Western blot analysis using antibodies against the phosphorylated form of indicated proteins. GAPDH was applied to confirm equal protein loading. At least 3 separate experiments were performed obtaining similar results. Data from one representative experiment are shown.

in pro-apoptotic cleaved caspase-3 level compared to control group ($p < 0.001$). Each polyphenol significantly inhibited X/XO-mediated increase of cleaved caspase-3. The inhibitory effects were observed more than in lower concentration of polyphenols (0.1 μg/ml) compared with higher one (10 μg/ml) (Fig. 3).

Discussion

Diets with high content of antioxidant polyphenols are associated with low prevalence of cardiovascular diseases (Karasu 2010; Andreadou et al. 2015). Quercetin and hydroxytyrosol, well known Mediterranean diet polyphenols, have cardioprotective properties during combat with oxidants (Rafehi et al. 2012; Chen et al. 2013; Miles et al. 2014). On the other hand, X/XO, a physiologically relevant system to generate superoxide and hydrogen peroxide, is also known to induce apoptosis and to mediate cardiomyocyte death (Seshadri et al. 2012; Miao et al. 2013). It has been suggested that upregulation of XO expression and/or XO activity accounts for, at least in part, myocardial hypoxia/reoxygenation oxidative injury (Zhang et al. 2015). To understand the cardioprotective action mechanism(s) of quercetin and hydroxytyrosol, we chose X/XO-induced, and oxidative stress-mediated cytotoxicity model and tested protective effects and molecular mechanisms involved in the treatment with quercetin or hydroxytyrosol in cardiomyocyte. As the key finding, we found that both quercetin and hydroxytyrosol are able to protect cardiomyocyte viability *via* inhibiting intracellular ROS generation and modulating the phosphorylation of survival or death signaling proteins when cardiomyocytes exposed to X/XO-induced oxidative stress.

Mitogen-activated protein kinases (MAPKs), including the extracellularly responsive kinases (ERKs), the stress-activated protein kinases (SAPKs) such as the c-Jun N-terminal kinases (JNKs), and the p38 MAPKs, have been found to play an important role in cardiac hypertrophy (Ge et al. 2013). While constitutive activation of the ERK 1/2 pathway significantly induces cardiac enlargement, the respective roles of the JNK and p38 pathways are controversial (Heineke and Molkentin 2006). Previously, it has been shown that xanthine oxidase plays an important role in in the regulation of two kinases, p38 kinase and ERKs (Kang et al. 2006). The activated p38 mitogen-activated protein kinase (p38 MAPK) phosphorylates its potential downstream targets such as MAP kinase-activated protein kinase-2 (MAPKAPK-2). We found that phosphorylated MAPKAPK-2, an index of p38 MAP kinase activity were significantly increased in X/XO-exposed cardiomyocytes, and both polyphenol significantly inhibited superoxide-induced MAPKAPK-2 phosphorylation. This is important because the ROS-mediated chronic activation of p38

MAPK and its downstream cascade has been implicated in myocyte hypertrophy and a wide spectrum of cardiac pathologies, and the inhibition of p38 MAPK may offer a therapeutic approach for cardiovascular disease as indicated previously (Kang et al. 2006; Bao et al. 2007; Scharf et al. 2013) .

On the other hand, Ras-Raf-MEK-ERK pathway is critically involved in cardiac proliferation (Scharf et al. 2013). We found that X/XO exposure resulted in increased phosphorylation of p44/42 MAPK (Erk1/2) in H9c2 cells. While quercetin, at low concentration, inhibited phosphorylation of both p44 and p42 (Erk1/2) against X/XO induction, Erk2 was slightly increased at the higher concentrations of polyphenolic compounds. In line with the general understanding of ROS and Erk1/2 regulation, it is well appreciated that the uncontrolled activation of Ras-Raf-MEK-ERK signaling triggers hypertrophic cardiomyopathy; but inhibiting the pathway also can render heart more vulnerable to stress-induced myocyte death (Heineke and Molkentin 2006; Wang 2007).

Heat shock proteins (Hsp) are a group of proteins that accumulate in the cells after a variety of physiological, environmental and pathological stresses. The phosphorylated heat shock protein 27 (Hsp27) has been shown to provide enhanced protection against disruption of cytoskeletal elements in various stress or pathological insults (Yu et al. 2012; Bali et al. 2014; Monda et al. 2014). While Hsp27 plays a crucial role as an antioxidant (Zhang et al. 2010; Bali et al. 2014), the transgenic mice with high levels of Hsp27 have been shown to develop cardiomyopathy (Zhang et al. 2010). In the present study, we demonstrated that X/XO exposure decreases the phosphorylation of Hsp27. While quercetin significantly and concentration-dependently inhibited X/XO-induced down-regulation of p-Hsp27, hydroxytyrosol led to further increase in the phosphorylation of Hsp27. A notable feature of the data presented in this study concerns
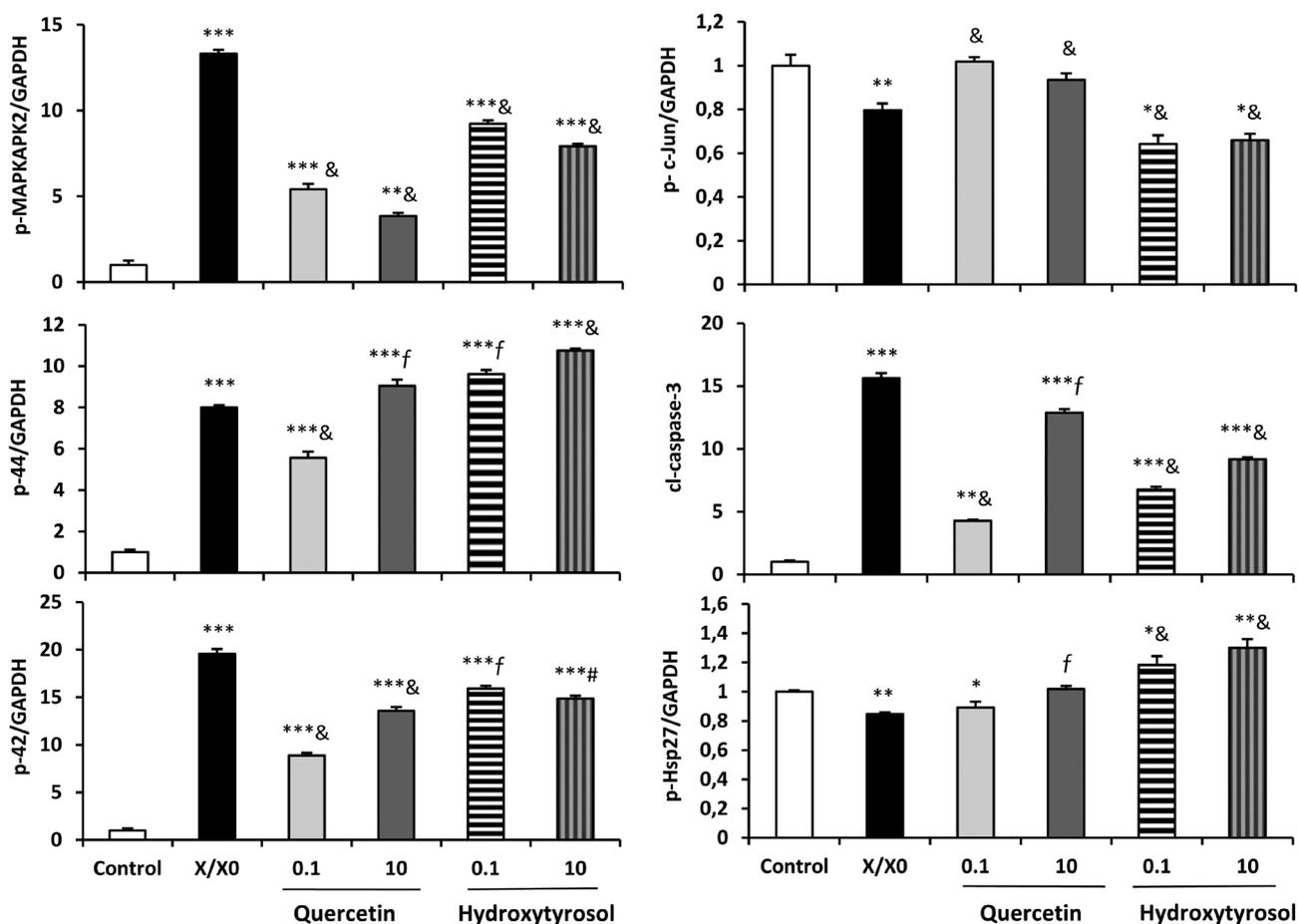

Figure 3. Western blot analysis of survival or death protein levels in H9c2 cells. The cells were pretreated with quercetin or hydroxytyrosol (0.1 and 10 μg/ml, 24 h) and then exposed to X/XO (0.5 mM/5 mU/ml) for 12 h. The results were expressed as the relative expression to GAPDH and plotted as the ratio of the control. All data are expressed as the means ± SD (ANOVA). $*$ $p < 0.05$, $**$ $p < 0.01$, $***$ $p < 0.001$ *vs.* Control; $^f p < 0.05$, $^\# p < 0.01$, $^\& p < 0.001$ *vs.* X/XO.

the nature of cardiomyocyte protecting ability of quercetin and hydroxytyrosol, which overcome X/XO-induced cell death and help to maintain survival through modulation of ERK1/2 and Hsp27 phosphorylation.

We further observed that the levels of active c-Jun levels were significantly reduced in X/XO-exposed cells. As a stress-induced signaling pathway, JNKs have both protective and pathological roles in cardiomyocyte function. In early studies performed in cultured neonatal cardiomyocytes, JNK activation, which phosphorylates c-Jun, led to a hypertrophic phenotype and also mediated cardiac remodeling (Ferrandi et al. 2004). In our toxicity model, the inhibition of p-c-Jun might be essential to maintain cellular homeostasis since the inhibition of c-Jun N-terminal kinase has been shown to inhibit cardiomyocyte apoptosis

and to decrease infarct size after myocardial ischemia and reperfusion (Daubney et al. 2014). Interestingly, while p-c-Jun reversed by quercetin, this factor was inhibited by hydroxytyrosol in X/XO-exposed cells more than in the cells exposed of X/XO alone. This seems to be not related with the contradictory effect of duration of preincubation because a recent study showed that the prolonged exposure (72 h) of H9c2 cells to this polyphenol leads to cytotoxicity while 24 h treatment contributes to protection and anti-apoptosis (Daubney et al. 2014).

As reported previously, the expression or phosphorylation of caspase-3 are increased in the presence of XO activating (hypoxia/reoxygenation) or superoxide generating systems (exposure to X/XO) in H9c2 (Costa et al. 2009; Wu et al. 2014). In accordance with this, we demonstrated that,
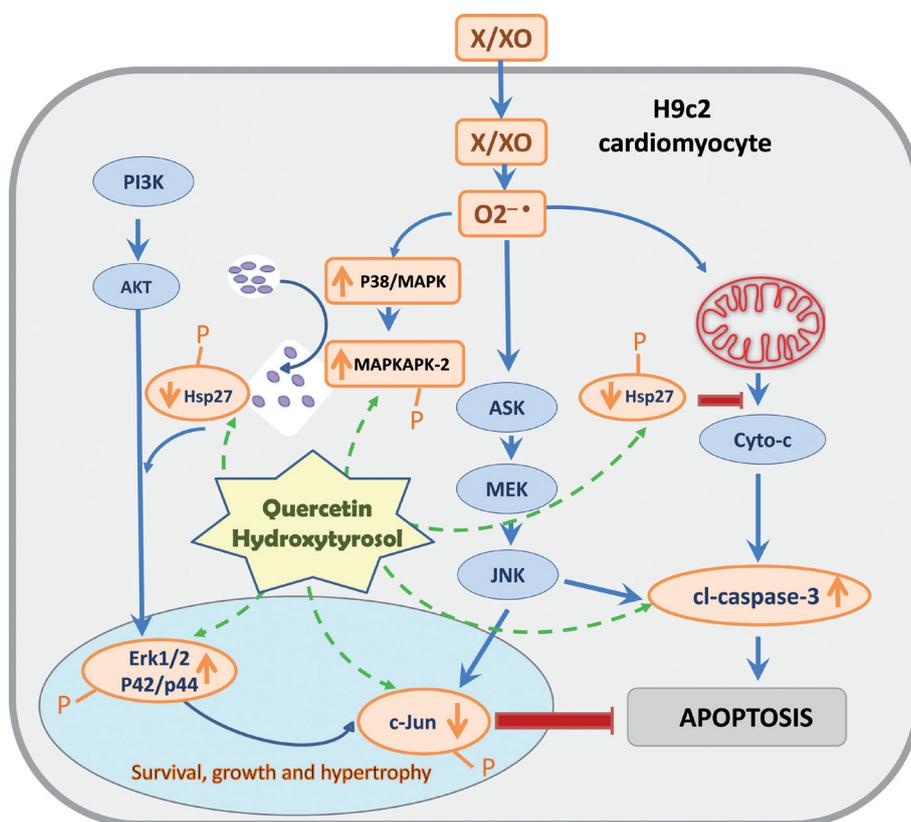
Figure 4. Quercetin and hydroxytyrosol inhibits X/XO-induced cardiomyocyte toxicity through the modulation of multiple stress-sensitive protein kinase cascades and transcription factors associated with cell survival and cell death. Full line arrows indicate intracellular targets of X/XO toxicity resulting in increase (up short arrows) or decrease (short down arrows) expression levels of signaling molecules. Broken line arrows show modulated pathways and signaling cascade by quercetin and hydroxytyrosol under X/XO-induced stress in H9c2 cells. MAPKAPK-2 is a protein kinase activated downstream of p38-MAPK which phosphorylates the small heat shock proteins Hsp27 is found to be critical in quercetin and hydroxytyrosol-induced cardiomyocyte protection against X/XO-induced toxicity. Hsp27 helps to maintain survival through modulation of Erk1/2 phosphorylation and inhibition of cytochrom c releasing from mithochondria. Phosphorylation of Jun increases transcription of the c-jun target genes, and active Erk 1/2 is found to increase c-jun transcription, which protects cells against X/XO-induced apoptosis. ROS increased levels by X/XO leads to activation of ASK-1, resulting in the activation of JNK and p38 pathways. The PI3K pathway, activated by many survival factors, leads to the activation of AKT, an important player in survival signaling. The effects of quercetin and hydroxytyrosol on signaling molecules, PI3K, AKT, ASK, MEK, JNK, Cyto-c need to be investigation under X/XO-induced oxidative stress conditions in cardiomyocytes. MAPKAPK2, mitogen-activated protein kinase-activated protein kinase 2; c-Jun, a signal-transducing transcription factor of AP-1 family; p44/42 MAPK (Erk1/2), mitogen-activated protein kinase 3 (MAPK3) is also known as extracellular signal-regulated kinase 1 (Erk1); MAPK1, mitogen-activated protein kinase 1 also known as extracellular signal-regulated kinase 2 (Erk2); JNK, c-Jun N-terminal kinases; ASK, apoptosis signal-regulating kinase; MEK, mitogen-activated protein (MAP)/extracellular signal-regulated kinase (Erk) kinase; AKT, protein kinase B; PI3K, phospholipid kinase phosphatidylinositol 3-kinase; Cyto-c, cytochrome c.

X/XO results in increased pro-apoptotic cleaved caspase-3 level in H9c2. Although this increase was significantly inhibited by each polyphenols, it was observed more in lower concentration of the polyphenols compared with higher one.

At present, we do not know the exact reason(s) for further up-regulation of p-Erk2 by both polyphenolic compounds, why hydroxytyrosol led to further increase in the phosphorylation of Hsp27 and why effects of two polyphenols on the most of parameters studied were concentration-dependent manner while in others did not in the presence of X/XO. In summary, these results may be point towards a ROS-survival protein feedback loop in which, at an early phase, X/XO generates ROS leading to activation of pMAPKAPK2, cl-caspase-3 that in turn transactivates p44/42 MAPK (Erk1/2) and inhibits p-cJun to partially reverse the apoptotic ability of X/XO. These changes may magnify the polyphenols, especially hydroxytyrosol, signal to finally activate the Hsp27and also p-44 phosphorylation for amplify a cardiomyocyte protection. On the other hand, under oxidative conditions, ERK1/2, JNK, and p38 as upstream regulators of the nuclear factor erythroid 2-related factor 2 (Nrf2) cascade has been shown to play crucial role for the activation of this cascade. The differences between cardiomyocyte responses to two polyphenols may be attributed to the complex signal transduction from polyphenols to Nrf2 or the different interplay between polyphenols and protective Nrf2 signaling in the presence of X/XO. Quercetin and hydroxytyrosol have been recently shown to display cytoprotective activity through the activation of Nrf2 pathway, scavenging of ROS, and inhibition of cellular death (Zou et al. 2012; Liu et al. 2015).

Conclusion

To the best of our knowledge, this is the first study to provide evidence that quercetin and hydroxytyrosol protect cardiomyocyes against X/XO-induced oxidative stress and toxicity *via* maintaining of cell viability and inhibition of ROS generation. The regulation of multiple stress-sensitive protein kinase cascades and transcription factors that promote cell proliferation, survival or apoptosis can in part underlie the observed effects of quercetin and hydroxytyrosol as summarized in Fig. 4.

Conflict of interest: The authors declare no conflict of interest.

References

Andreadou I., Benaki D., Efentakis P., Bibli S. I., Milioni A. I., Papachristodoulou A., Zoga A., Skaltsounis A. L., Mikros E., Iliodromitis E. K. (2015): The natural olive constituent oleuropein induces nutritional cardioprotection in normal and cholesterol-fed rabbits: Comparison with preconditioning. Planta Med. **8,** 655–663

Bali E. B., Ergin V., Rackova L., Bayraktar O., Küçükboyacı N., Karasu C. (2014): Olive leaf extracts protect cardiomyocytes against 4-HNE-induced toxicity in vitro: Comparison with oleuropein, hydroxytyrosol and quercetin. Planta Med. **80,** 984–992
http://dx.doi.org/10.1055/s-0034-1382881

Bao W., Behm D. J., Nerurkar S. S., Ao Z., Bentley R., Mirabile R. C., Johns D. G., Woods T. N., Doe C. P., Coatney R. W., Ohlstein J. F., Douglas S. A., Willette R. N., Yue T. L. (2007): Effects of p38 MAPK Inhibitor on angiotensin II-dependent hypertension, organ damage, and superoxide anion production. J. Cardiovasc. Pharmacol. **49,** 362–368
http://dx.doi.org/10.1097/FJC.0b013e318046f34a

Chen J. Y., Hu R. Y., Chou H. C. (2013): Quercetin-induced cardioprotection against doxorubicin cytotoxicity. J. Biomed. Sci. **20,** 95
http://dx.doi.org/10.1186/1423-0127-20-95

Costa V. M., Silva R., Ferreira R., Amado F., Carvalho F., de Lourdes Bastos M., Carvalho R. A., Carvalho M., Remião F. (2009): Adrenaline in pro-oxidant conditions elicits intracellular survival pathways in isolated rat cardiomyocytes. Toxicology **257,** 70–79
http://dx.doi.org/10.1016/j.tox.2008.12.010

Cumaoğlu A., Ari N., Kartal M., Karasu C. (2011a): Polyphenolic extracts from Olea europaea L protect against cytokine-induced β-cell damage through maintenance of redox homeostasis. Rejuvenation Res. **14,** 325–334
http://dx.doi.org/10.1089/rej.2010.1111

Cumaoğlu A., Rackova L., Stefek M., Kartal M., Maechler P., Karasu C. (2011b): Effects of olive leaf polyphenols against H_2O_2 toxicity in insulin secreting β-cells. Acta Biochim. Pol. **58,** 45–50.

Daubney J., Bonner P. L., Hargreaves A. J., Dickenson J. M. (2014): Cardioprotective and cardiotoxic effects of quercetin and two of its in vivo metabolites on differentiated H9c2cCardiomyocytes. Basic Clin. Pharmacol. Toxicol. **116,** 96–109
http://dx.doi.org/10.1111/bcpt.12319

Du Y., Guo H., Lou H. (2007): Grape seed polyphenols protect cardiac cells from apoptosis via induction of endogenous antioxidant enzymes. J. Agric. Food Chem. **55,** 1695–1701
http://dx.doi.org/10.1021/jf063071b

Durot I., Maupoil V., Ponsard B., Cordelet C., Vergely-Vandriesse C., Rochette L., Athias P. (2000): Oxidative injury of isolated cardiomyocytes: dependence on free radical species. Free Radic. Biol. Med. **29,** 846–857
http://dx.doi.org/10.1016/S0891-5849(00)00382-8

Ergin V., Hariry R. E., Karasu C. (2013): Carbonyl stress in aging process: role of vitamins and phytochemicals as redox regulators. Aging Dis. **4,** 276–294
http://dx.doi.org/10.14336/AD.2013.0400276

Ferrandi C., Ballerio R., Gaillard P., Giachetti C., Carboni S., Vitte P. A., Gotteland J. P., Cirillo R. (2004): Inhibition of c-Jun N-terminal kinase decreases cardiomyocyte apoptosis and infarct size after myocardial ischemia and reperfusion in anaesthetized rats. Br. J. Pharmacol. **142,** 953–960

http://dx.doi.org/10.1038/sj.bjp.0705873

Ge Y., Pan S, Guan D., Yin H., Fan Y., Liu J., Zhang S., Zhang H., Feng L., Wang Y, Xu R., Yin J. Q. (2013): MicroRNA-350 induces pathological heart hypertrophy by repressing both p38 and JNK pathways. Biochim. Biophys. Acta **1832,** 1–10
http://dx.doi.org/10.1016/j.bbadis.2012.09.004

Heineke J., Molkentin J. D. (2006): Regulation of cardiac hypertrophy by intracellular signalling pathways. Nat. Rev. Mol. Cell Biol. **7,** 589–600
http://dx.doi.org/10.1038/nrm1983

Hu T., He X. W., Jiang J. G., Xu X. L. (2014): Hydroxytyrosol and its potential therapeutic effects. J. Agric. Food Chem. **62,** 1449–1455
http://dx.doi.org/10.1021/jf405820v

Incani A., Deiana M., Corona G., Vafeiadou K., Vauzour D., Dessì M. A., Spencer J. P. (2010): Involvement of ERK, Akt and JNK signalling in H2O2-induced cell injury and protection by hydroxytyrosol and its metabolite homovanillic alcohol. Mol. Nutr. Food Res. **54,** 788–796
http://dx.doi.org/10.1002/mnfr.200900098

Janjic D., Wollheim C. B. (1992): Islet cell metabolism is reflected by the MTT (tetrazolium) colorimetric assay. Diabetologia **35,** 482–485
http://dx.doi.org/10.1007/BF02342448

Kang S. M., Lim S., Song H., Chang W., Lee S., Bae S. M., Chung J. H., Lee H., Kim H. G., Yoon D. H., Kim T. W., Jang Y., Sung J. M., Chung N. S., Hwang K. C. (2006): Allopurinol modulates reactive oxygen species generation and Ca2+ overload in ischemia-reperfused heart and hypoxia-reoxygenated cardiomyocytes. Eur. J. Pharmacol. **535,** 212–219
http://dx.doi.org/10.1016/j.ejphar.2006.01.013

Karasu C. (2010): Glycoxidative stress and cardiovascular complications in experimentally-induced diabetes: effects of antioxidant treatment. Open Cardiovasc. Med. J. **4,** 240–256
http://dx.doi.org/10.2174/1874192401004010240

Khurana S., Venkataraman K., Hollingsworth A., Piche M., Tai T. C. (2013): Polyphenols: benefits to the cardiovascular system in health and in aging. Nutrients **5,** 3779–3827
http://dx.doi.org/10.3390/nu5103779

Liu C. M., Ma J. Q., Xie W. R., Liu S. S., Feng Z. J., Zheng G. H., Wang A. M. (2015): Quercetin protects mouse liver against nickel-induced DNA methylation and inflammation associated with the Nrf2/HO-1 and p38/STAT1/NF-κB pathway. Food Chem. Toxicol. **82,** 19–26
http://dx.doi.org/10.1016/j.fct.2015.05.001

Miao Y., Zhou J., Zhao M., Liu J., Sun L., Yu X., He X., Pan X., Zang W. (2013): Acetylcholine attenuates hypoxia/ reoxygenation-induced mitochondrial and cytosolic ROS formation in H9c2 cells via M2 acetylcholine receptor. Cell. Physiol. Biochem. **31,** 189–198
http://dx.doi.org/10.1159/000343360

Miles S. L., McFarland M., Niles R. M. (2014): Molecular and physiological actions of quercetin: need for clinical trials to assess its benefits in human disease. Nutr. Rev. **72,** 720–734
http://dx.doi.org/10.1111/nure.12152

Monda M., Messina G., Scognamiglio I., Lombardi A., Martin G. A., Sperlongano P., Porcelli M., Caraglia M., Stiuso P. (2014): Short-term diet and moderate exercise in young overweight men modulate cardiocyte and hepatocarcinoma survival by oxidative stress. Oxid. Med. Cell Longev. **2014,** 131024

http://dx.doi.org/10.1155/2014/131024

Rafehi H., Ververis K., Karagiannis T. C. (2012): Mechanisms of action of phenolic compounds in olive. J. Diet. Suppl. **9,** 96–109
http://dx.doi.org/10.3109/19390211.2012.682644

Rosenkranz A. R., Schmaldienst S., Stuhlmeier K. M., Chen W., Knapp W., Zlabinger G. J. (1992): Microplate assay for the detection of oxidative products using **2,**7-dichlorofluorescein-diacetate. J. Immunol. Methods **156,** 39–45
http://dx.doi.org/10.1016/0022-1759(92)90008-H

Scharf M., Neef S., Freund R., Geers-Knörr C., Franz-Wachtel M., Brandis A., Krone D., Schneider H., Groos S., Menon M. B., Chang K. C., Kraft T., Meissner J. D., Boheler K. R., Maier L. S., Gaestel M., Scheibe R. J. (2013): Mitogen-activated protein kinase-activated protein kinases 2 and 3 regulate SERCA2a expression and fiber type composition to modulate skeletal muscle and cardiomyocyte function. Mol. Cell. Biol. **33,** 2586–2602
http://dx.doi.org/10.1128/MCB.01692-12

Seshadri G., Che P. L., Boopathy A. V., Davis M. E. (2012): Characterization of superoxide dismutases in cardiac progenitor cells demonstrates a critical role for manganese superoxide dismutase. Stem Cells Dev. **21,** 3136–346
http://dx.doi.org/10.1089/scd.2012.0191

Wang Y. (2007): cMitogen-activated protein kinases in heart development and diseases. Circulation **116,** 1413–1423
http://dx.doi.org/10.1161/CIRCULATIONAHA.106.679589

Wu X., Zhou S., Zhu N., Wang X., Jin W., Song X., Chen A. (2014): Resveratrol attenuates hypoxia/reoxygenation-induced Ca2+ overload by inhibiting the Wnt5a/Frizzled-2 pathway in rat H9c2 cells. Mol. Med. Rep. **10,** 2542–2548
http://dx.doi.org/10.3892/mmr.2014.2488

Yu Y., Liu M., Zhang L., Cao Q., Zhang P., Jiang H., Zou Y., Ge J. (2012): Heat shock transcription factor 1 inhibits H2O2-induced cardiomyocyte death through suppression of high-mobility group box 1. Mol. Cell. Biochem. **364,** 263–269
http://dx.doi.org/10.1007/s11010-012-1226-x

Zhang X., Min X., Li C., Benjamin I. J., Qian B., Zhang X., Ding Z., Gao X., Yao Y., Ma Y., Cheng Y., Liu L. (2010): Involvement of reductive stress in the cardiomyopathy in transgenic mice with cardiac-specific overexpression of heat shock protein 27. Hypertension **55,** 1412–1417
http://dx.doi.org/10.1161/HYPERTENSIONAHA.109.147066

Zhang Y. S., Liu B., Luo X. J., Zhang J. J., Li N. S., Ma Q. L., Jiang J. L., Li Y. J., Li Q., Peng J. (2015): A novel function of nuclear non-muscle myosin regulatory light chain in promotion of xanthine oxidase transcription after myocardial ischemia/reperfusion. Free Radic. Biol. Med. **83,** 115–128
http://dx.doi.org/10.1016/j.freeradbiomed.2015.02.013

Zou X., Feng Z., Li Y., Wang Y., Wertz K., Weber P., Fu Y., Liu J. (2012): Stimulation of GSH synthesis to prevent oxidative stress-induced apoptosis by hydroxytyrosol in human retinal pigment epithelial cells: activation of Nrf2 and JNK-p62/SQSTM1 pathways. J. Nutr. Biochem. **23,** 994–1006
http://dx.doi.org/10.1016/j.jnutbio.2011.05.006