RESVERATROL SUPLEMENTATION AT OLD AGE REVERTS CHANGES ASSOCIATED WITH AGING IN INFLAMMATORY, OXIDATIVE AND APOPTOTIC MARKERS IN RAT HEART

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ABSTRACT

Purpose

Aging is known to play a critical role in the etiopathogenesis of several diseases. Among them, cardiovascular disorders are especially relevant since they are becoming the first cause of death in western countries. Resveratrol is a polyphenolic compound that has been shown to exert beneficial effects at different levels, including neuronal and cardiovascular protection. Those effects of resveratrol are related, at least in part, to its antioxidant and anti-inflammatory properties. In the current investigation we were interested in exploring whether the positive effects of resveratrol at cardiac level were taking place even when the supplementation started in already old animals.

Methods

Old male rats were supplemented with resveratrol during 10 weeks. Using RT-PCR, we analyzed the effects of resveratrol supplementation on the expression of different genes related to inflammation, oxidative stress and apoptosis in rat heart.

Results

Resveratrol reverted the age-related changes in inflammatory, oxidative and apoptotic markers in the rat heart. Among others, the expression of two major inflammatory markers, INF-γ and TNF-α and two oxidative markers, heme oxygenase and nitric oxide synthase, were increased with aging, and resveratrol supplementation reduced their levels to those observed in the heart of young animals. Moreover, age-related changes in apoptotic markers in rat heart were also reverted by resveratrol treatment.

Conclusion

Our results suggest that resveratrol might exert beneficial effects as an anti-aging compound in order to revert age-related changes in cardiac function.

KEYWORDS

Resveratrol, aging, heart, inflammation, oxidation, apoptosis

INTRODUCTION

Cardiovascular diseases are becoming the first cause of death in western countries, and in the next two decades the proportion of total deaths worldwide due to cardiovascular diseases within the elderly population (>70 yr) will be 40% [1]. Although long- term exposure to risk factors, such as those related to lifestyle (diet, physical inactivity…), plays a major role in the etiopathogenesis of cardiac disorders, aging itself is considered to be the major determinant for developing cardiac diseases [2]. Thus, the increased life expectancy has as a direct consequence: a higher incidence in age-related diseases, in particular those associated with the cardiovascular system.

During the last decades different investigations have stressed the relevance of inflammation and oxidative stress both in heart aging and in the onset and development of cardiac diseases, such as heart failure, cardiac hypertrophy and diabetic cardiomyopathy (reviewed in [3]). Age-related increases in those processes lead to reduced cellular survival and cardiac dysfunction. Moreover, inflammation and oxidative stress are also believed to play an important role in age- related changes in the vascular system, in particular in the reduction in the endothelium-dependent relaxation and increases in endothelium-dependent contraction [4]. Cytokines are major signaling proteins involved in immunity, inflammation and hematopoiesis, among other processes. During aging, a shift in the cytokine profile in blood occurs, with increases in inflammatory substances such as tumor necrosis factor- alpha (TNF-α) and interleukins (IL) 1, 2 and 6, as well as decreases in anti-inflammatory substances like IL-10 [5]. Regarding oxidative stress, mitochondrial reactive oxygen species (ROS) production is considered the most important source of cellular ROS in healthy

tissues since the main free radical generator, the electron transport chain, is located at the inner mitochondrial membrane. Together with ROS, reactive nitrogen species (RNS) also play an important role in the disruption of redox signaling and in molecular damage. RNS includes the nitric oxide radical (NO), which is mainly generated by NO synthases (NOS), and other compounds originated by the reaction of NO with ROS, which results in peroxynitrites. During aging, the age-associated organ dysfunction has been partly related with the accumulation of damage induced by ROS and NOS [6].

Eventually, inflammatory and oxidative processes have been described to contribute to mitochondrial dysfunction that may trigger apoptotic signaling [7,8]. According to this, an over-activation of the apoptotic pathways has been observed in age-related diseases, including neurodegenerative processes and cardiovascular events such as ischemia/ reperfusion [9,10].

Different strategies have been used trying to improve aging and postpone the onset of age-related disorders. Among them, nutritional intervention is very popular, including both dietary restriction and dietary supplementation. Dietary restriction (DR) has been used as a model for investigating the mechanisms underlying the aging process for many years and it has described to increase lifespan and reduce the incidence of age-related diseases in different species [11,12]. Interestingly, the positive effects of DR have been described to take place even when restriction is initiated in middle-aged animals [11]. On the other hand, one of the most popular dietary supplements that has been used due its beneficial effects on age-related detrimental changes is resveratrol [13]. Resveratrol (3,5,4′-trihydroxy-trans-stilbene) is a polyphenolic phytoalexin present in some food products including grapes and berries. The relevance of

resveratrol exponentially rose when it was considered as a potential DR mimetic, being identified as an activator of Sirt1 [14]. Nevertheless, sirt1 is not the only target of resveratrol. It has been described to inhibit cyclooxygenase 1, cAMP phosphodiesterases, and to interact with the estrogen receptor alpha [15]. The beneficial effects of resveratrol are diverse, including suppression of cancer cell growth, neuroprotection and protection against age-related cardiovascular dysfunction [15]. However, most of the studies on resveratrol have been performed in young/ adult animals, when the deleterious effects of aging are still missing. In the current investigation we were interested in exploring whether supplementation with resveratrol in old animals reverted the age-related changes in different parameters covering various aspects of inflammation, oxidation and apoptosis in the rat heart. We observed that when old animals were supplemented with resveratrol, the expression of genes related to those processes were reverted to levels observed in young animals. Moreover, those effects were related, at least in part, by the changes in SIRT1 levels. Our results suggest that resveratrol would exert beneficial effects on cardiac function even when supplementation starts at advanced age.

MATERIAL AND METHODS

Animals and treatment

This study was carried out in accordance with the guidelines for Ethical Care of Experimental Animals of the European Union, approved by the Ethical Committee for Animal Studies of the Complutense University (Madrid, Spain). Young (2 month-old) and old (24 month-old) male Wistar rats were compared in this study. All rats were housed in standard conditions and fed a standard

laboratory rat diet and water *ad libitum*. One group of old rats was treated with resveratrol at dosages of 10 mg/ kg per day during 10 weeks. In humans, this dosage would correspond to approximately to 100mg/ day, assuming an average human body weight of 60Kg. This would correspond to a mild dose in humans. Several studies in humans have shown that resveratrol is well tolerated with no marked toxicity [16,17], and only the daily administration at very high doses of 2.5- 5g per day for 29 days caused mild to moderate gastrointestinal symptoms [18]. Using allometric scaling [19], the human equivalent dosage to the one we used in old rats in the current investigation, based on body surface area, is 1.61 mg / kg / day. In the present study, old rats were treated with resveratrol at doses of 10 mg / kg / day for 10 weeks.
The second group of rats was left untreated as a control group. Resveratrol was obtained from Actafarma Laboratories, Madrid, Spain. It was dissolved in absolute ethanol and added to the drinking water in a final ethanol concentration of 0.1%. Resveratrol solution was prepared according to water intake of the animals, in order to ensure that they received the right dosage. Water bottles were covered with aluminium foil for protection from light, and the drinking fluid was changed every day. Untreated animals received same ethanol concentration in tap water. Animals were sacrificed by decapitation and the heart (ventricles) removed.

RNA isolation and RT-PCR

RNA was isolated from heart samples using the method described by Chomczynski and Sacchi [20], using the TRI Reagent Kit (Molecular Research Center, Inc., Cincinnati, OH), following the manufacturer's protocol. The purity

of the RNA was estimated by 1.5% agarose gel electrophoresis, and RNA concentration was determined by spectrophotometry (260 nm). Reverse transcription of 2 µg RNA for cDNA synthesis was performed using the Reverse Transcription System (Promega, Madison, WI) and a pd(N)6 random hexamer. RT-PCR was performed in an Applied Biosystems 7300 apparatus using the SYBR Green PCR Master Mix (Applied Biosystems, Warrington, UK) and 300-nM concentrations of specific primers (Table 1). The thermocycling profile conditions used were: 50°C for 2 m, 95°C for 10 m, 95°C for 15 s, 60°C for 1 m, 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. For the normalization of cDNA loading in the PCR reaction, the amplification of the 18S rRNA for every sample was used. Relative changes in gene expression were calculated using the 2-ΔΔCT method [21].

Purification of total protein extracts and western blotting

In order to obtain the total protein extracts, hearts were homogenized in RIPA buffer (1:10 w/v) (50 mM TRIS, 150 mM NaCl, 1 % triton 9100, 0.5 % Sodium deoxycholate, 0.1 % SDS) in the presence of protease inhibitors (protease inhibitor cocktail (Sigma)) and incubated 30 min on ice. After centrifugation at 10000g for 30 min, the supernatants were collected. Samples were aliquoted and stored at -80ºC until use. The protein concentration of the different preparations was determined by the Lowry method (Lowry et al. 1951).
Total heart samples (75 µg protein) were separated on 4–15 % MiniPROTEAN TGX^{TM} gels (Bio-Rad) and transferred to PVDF membranes (Millipore). SIRT 1 protein levels were detected by using a monoclonal mouse antibody (1:500 dilution; ab110304 Abcam). ß-Actin (monoclonal mouse antibody. 1:10000

dilution, A2228 Sigma) was used as loading control. Secondary anti-mouse antibody was used at 1:5000 dilution. Chemiluminescent reaction was performed using ECL plus® (GE Healthcare, Amersham) and visualized in a GeneGnomeXRQ (Syngene). In order to estimate the levels of SIRT1, the intensity of the bands was quantified using the specific Gene Snap software (Syngene)

Statistical analysis

All data are reported as mean ± standard error of the mean (SEM) using from 4 (old animal group) to 6 (young group and old resveratrol treated group) independent preparations, except when indicated. The results were analyzed by ANOVA followed by Fisher's test. Statistical analyses were carried out using the Graphpad Prism Software. The minimum level of statistical significance was set at $p<0.05$ in all analyses.

RESULTS

SIRT1 levels were increased in the heart of old rats after resveratrol treatment

During aging SIRT1 levels were reduced by 20% in rat heart although this reduction was not statistically significant ($p=0.50$) (Figure 1). When old animals were subjected to resveratrol treatment, SIRT1 protein levels were 20% higher than old control counterparts ($p=0.38$), reaching protein levels similar to those observed in young rats.

Resveratrol reverted the age-related changes in the transcription of inflammation- related genes in the heart of old rats

One of the main hallmarks of aging is a chronic inflammation state. In the current investigation, we did observe such pro- inflammatory state in the heart of old rats (Figure 2). A significant age-related increase in the transcription of pro-inflammatory cytokines INF-γ and TNF-α ($p=0.04$ and $p=0.03$, respectively) was observed. Such increase was especially important in the case of TNF-α, with a 2.2 –fold enhancement. We also investigated one of the main transcription factors related to pro-inflammatory cytokines, NFκB. Similarly to what we observed in pro-inflammatory cytokines, the expression of NFκB2 (p52) increased by 45% with age in rat heart, although it did not reach statistical significance ($p=0.29$). In addition, the transcription levels of the anti-inflammatory cytokine IL-10 were 25% lower in the heart of old rats than in young animals, although such reduction was not statistically significant ($p=0.43$). On the other hand, resveratrol treatment led to a significant reduction in the levels of pro- inflammatory markers INF-γ and TNF-α ($p<0.05$ and $p=0.08$), and it also reduced the age-related enhancement in p52 transcription levels, yet again, not significantly ($p=0.60$). Conversely, resveratrol increased the transcription of IL-10 ($p=0.39$), reaching comparable levels to those observed in young animals.

Resveratrol treatment reduced the age-related increase in the transcription of oxidation- related genes in the heart of old rats

Similarly to what we observed with inflammatory markers, the heart of aged rats showed higher transcription levels of oxidative stress markers (Figure 3).

Expression of HO-1 was strongly induced with aging ($p < 0.001$) and although resveratrol treatment significantly reduced such an increase ($p = 0.0032$), it did not completely revert it. Similarly, HO-2 expression was also enhanced with aging. However, this increase was not statistically significant ($p=0.11$). Nevertheless, resveratrol significantly reduced the expression levels of HO-2 in the heart of old rats ($p=0.18$). We observed and age-related increase in the expression of both endothelial (eNOS; $p=0.06$) and inducible (iNOS; $p=0.24$) NOS. Moreover, the expression of iNOS was significantly reduced in old animals when they were subjected to resveratrol treatment ($p \leq 0.05$) to levels similar to what we observed in young rats, whereas eNOS expression levels, although diminished, did not change significantly ($p=0.29$).

The transcription of genes related to apoptosis was increased in the heart of aged animals and reverted by resveratrol treatment

Inflammation and oxidative stress may induce the activation of the apoptotic pathways, which would eventually lead to cell death. Enhancement in apoptosis would be particularly relevant in tissues containing post-mitotic cells such as the heart, since the tissue function might be compromised. Therefore, we investigated the expression of four different genes related to both, caspase-dependent and independent apoptotic pathways (Figure 4). We observed that with the exception of XIAP ($p=0.07$), the transcription of AIF, BAD and Bcl-2 markers was significantly increased in the heart of old animals ($p \leq 0.05$), being the increase in AIF the most important one (1.9- fold increase; $p<0.0064$). Similarly to what we observed in inflammatory and oxidative factors, resveratrol treatment reduced the expression levels of all the analyzed apoptotic markers.

However, such reduction did not reach statistical significance for any of the markers, AIF (p=0.08), BAD (p=0.20), Bcl-2 (p=0.07) and XIAP levels (p=0.35).

DISCUSSION

Nowadays, cardiovascular diseases are the first cause of morbidity and mortality in western countries. Thus, searching for new strategies that may help to delay the development of cardiovascular diseases is of great interest. One of the main risk factors for cardiovascular dysfunction is aging [3], and different approaches have been used in order to improve the age-related decline in cardiovascular function and delay the onset of cardiovascular diseases. Among them, supplementation with resveratrol has been described to exert beneficial effects on the cardiovascular system [22,23]. However, whether resveratrol has beneficial effects once the age-related deterioration has taken place, is unknown. In the current investigation, we analyzed whether resveratrol supplementation had beneficial effects on the cardiovascular system, even when the treatment starts in animals that are already old.

Resveratrol acts as an activator of different signaling pathways, but the activation of SIRT1 seems to critical [24,25]. Moreover, it has been reported that resveratrol induces the expression of SIRT1 as well [26,27]. SIRT1 belongs to the sirtuin family of proteins and it is the most studied one in mammals. Sirtuins are NAD^+ -dependent protein deacetylases that regulate key cellular processes such as cell cycle, apoptosis or inflammation. Together with their role as transcription regulators through histone deacetylation, sirtuins are known to have different targets in cytosol and mitochondria, including, among others, p53, FoxO transcription factors, PGC-1α and NF-κB [28]. Sirtuins also activates

AMP-activated protein kinase (AMPK) [29], which is linked to the regulation of the mammalian target of rapamycin (mTOR) pathway with important consequences at cardiac level [30-32]

Different studies have shown that during aging or under specific pathological conditions, such as diabetes, sirtuin expression and activity are reduced [26,33,34]. Thus, the beneficial effects of resveratrol in relation to aging and the onset and progression of various diseases are likely to be related to its role as activator of sirtuins. Accordingly and in agreement with previous investigations [33,34], we observed that SIRT1 protein levels tended to be lower in aged animals and, as expected, resveratrol treatment reverted SIRT1 levels to those observed in young animals.

Age-related cardiac dysfunction has been associated with an increased in inflammatory processes (reviewed in [3]). In agreement with that, the inflammatory markers analyzed in the current study indicated a pro-inflammatory state in the heart of aged rats. In cardiac tissue, chronic inflammation is particularly relevant, since it has been suggested to significantly contribute to heart failure [35,36]. In particular, the increase in TNF-α levels has been related with cardiac hypertrophy and dilated cardiomyopathy [37]. In old animals, we have observed an increased in the pro- inflammatory markers INF-γ and TNF-α, together with a reduction in the levels of anti-inflammatory cytokines like IL-10. The enhancement of pro- inflammatory cytokines levels was related with an age-related increase tendency in the expression of p52, one of the proteins constituting homodimers and heterodimers with other NFκB proteins. NFκB is a complex transcription factor that is involved in a number of

299 physiological processes, including inflammation, oxidative stress and cell
300 survival [38]. NFκB is chronically elevated in the aging process and in many
301 age-related disorders including cardiovascular diseases [39].
302
303 Considering the described anti-inflammatory effects of sirtuins and the effect of
304 resveratrol supplementation on SIRT1 levels in the heart of our animals, we
305 expected to observe a reversion of the inflammatory markers in old animals
306 treated with resveratrol. The expression levels of pro-inflammatory cytokines
307 were actually reduced, reaching those observed in young animals. At the same
308 time, the reduction in the anti-inflammatory IL-10 observed in old animals was
309 reverted by resveratrol treatment. However, resveratrol treatment did not
310 change significantly p52 expression levels. Resveratrol has been described to
311 inhibit NFkB signaling by suppressing the activity of Ikß kinase (IKKß) [40].
312 Activation of IKKß is required for p52 activation, which suggests that the effect
313 of resveratrol might not be dependent on changes on p52 expression levels but
314 rather on its activity. In sum, our results support previous investigations showing
315 that resveratrol prevent inflammation in the cardiovascular system [41-43].
316 Together with inflammatory markers, we investigated the expression levels of
317 two enzymes related with oxidative stress: heme oxygenase (HO) and nitric
318 oxide synthase (NOS). The beneficial effects of resveratrol have been
319 described to be related with a lower ROS generation, which would be
320 responsible, at least in part, for its anti-oxidant effects [41,42]. Heme
321 oxigenases 1 and 2 are the inducible and constitutive isoforms respectively.
322 Both catalyze the degradation of heme to biliverdin, free iron, and carbon
323 monoxide (CO). HO-1 can be induced by several factors, including oxidative

stress, and HO-2, although it is constitutively expressed, may be regulated differently under certain conditions [44,45]. Both HO isoforms are believed to play an important role in the cellular antioxidant defense [46,47]. Moreover, the HO system has been described to have an important anti-inflammatory role [48]. In contrast with a recent study showing a decline in the levels of HO-1 and HO-2 and in the total HO activity in female rats with aging [49], we observed an age-related increase in the mRNA levels of both HO-1 and HO-2. However, it has been described that estrogens play an important role in the regulation of HO activity [49-51], suggesting that the pattern of age-related changes in HO expression in male and females animals may differ. In fact, a previous study in our laboratory showed that HO-1 levels were increased with aging in the heart of male mice [52], in agreement with the current results in male rats. The increase in HO levels might indicate an attempt to compensate an enhancement of oxidative stress and inflammation in the heart of old animals. We observed that resveratrol supplementation in old rats totally or partially blocked the age-related increase in HO transcription levels. Reported effects of resveratrol on HO levels are contradictory. Thus, despite it has been reported to induce HO-1 expression in cell cultures [53], supplementation with resveratrol has been described to revert increases in HO-1 levels observed in retina of diabetic rats [54] as well as to inhibit metastasis of lung adenocarcinoma cells by suppressing HO-1 activity [55]. In agreement with these studies, we observed that the HO-1 and HO-2 transcription levels were lower in the heart of resveratrol-supplemented rats than in aged control animals, suggesting that resveratrol supplementation might reduce oxidative stress in those animals.

348 Regarding NOS, we investigated the inducible (iNOS) and the constitutive
349 (eNOS) isoforms. Nitric oxide (NO) is generated by the activity of NOS, which
350 converts L-arginine into L-citrulline. NO bioavailability is critical for vascular
351 function and hence age-related reduction on this parameter is considered as a
352 main risk factor for developing cardiovascular diseases such as hypertension,
353 which may lead to cardiac failure [56]. Different studies have described
354 decreases, increases and even no changes in eNOS expression with aging
355 [56]. In the current investigation we have observed that both eNOS and iNOS
356 expression were higher in aged animals than in young counterparts. An
357 increase in iNOS expression has been previously described in the liver, heart
358 and pancreas of the senescence-accelerated mice model SAMP8 [52,57,58] as
359 well as in pancreas and heart of aged animals [52,59]. Increased iNOS
360 expression has been suggested to play an important role in reducing eNOS
361 activity and NO bioavailability [60], and it has been related to atherosclerosis
362 development, since iNOS has been described to be the main generator of free
363 radicals in the atheromatous plaques [61,62]. Moreover, an age-related
364 uncoupling of eNOS occurs with aging, leading to higher superoxide production
365 and peroxynitrate generation [56,63]. Thus, the higher levels of both isoforms of
366 NOS in the heart of aged animals suggest that cardiac tissue is subjected to an
367 increased oxidative and nitro-oxidative stress in those animals. Similarly to what
368 we observed in HO-1 and HO-2, resveratrol treatment reverted the age-related
369 increase in NOS isoforms, supporting the antioxidant and protective effect of
370 resveratrol on the aged heart as previously reported [34,41].

371 Together with the evidences of inflammation being activated through
372 enhancements in oxidative stress, there are several reports suggesting a cross-

373 talk between oxidative stress, inflammation and initiation of apoptotic events,
374 particularly in the cardiovascular system [7,8,64,65]. Age-related enhancement
375 in apoptosis has been related to cell loss in several tissues, including the heart,
376 leading to cardiac dysfunction and cardiomyopathies [66,67]. In the current
377 investigation, we observed that one of the pro-apoptotic factors in the caspase-
378 dependent pathway, BAD, was increased during aging. BAD is one of the pro-
379 apoptotic “Bcl-2 family” proteins that promote permeabilization of the
380 mitochondrial membrane and the release of cytochrome c. We also observed a
381 very significant increase, almost 2.5 fold, in the levels of AIF expression. This
382 pro-apoptotic factor is released from mitochondria and promotes apoptosis
383 through nuclear DNA fragmentation in a caspase- independent pathway,
384 stressing an age-related pro-apoptotic state in the rat heart. However, at the
385 same time, we observed that the expression of the anti-apoptotic protein Bcl-2,
386 which binds to pro-apoptotic proteins to inhibit mitochondrial permeabilization,
387 was significantly increased in old animals, as well as the levels of the caspase
388 inhibitor XIAP. These results suggest that the age-related increase in pro-
389 apoptotic factors would be counteracted by the enhancement of anti-apoptotic
390 factors of the caspase-dependent pathway. The augmented levels of anti-
391 apoptotic factors are likely a protective mechanism that would be important in
392 tissues with primarily post-mitotic cells like the heart, where cell loss would
393 greatly compromise their functionality. We have actually observed that in the
394 liver of these animals, which is a mitotic tissue, the expression of pro-apoptotic
395 proteins was increased 20-35% while the expression of anti-apoptotic proteins
396 like XIAP experienced a 20% reduction during aging (Torregrosa-Muñumer,
397 unpublished results), suggesting a different profile of apoptosis in heart and

liver. These differences in apoptosis when analyzing mitotic and post-mitotic tissues have been previously reported [68-70]. The effect of resveratrol treatment on the expression of pro- and anti-apoptotic markers was similar to the one observed in inflammatory and oxidative markers: it reverted the age-related changes in all cases.

Since resveratrol treatment led to a general reduction in inflammatory and oxidative markers in aged hearts, it was expected that its effect on pro-apoptotic markers would be also similar. The apoptotic response of a cell is regulated by the relative balance of pro- and anti-apoptotic proteins. Cellular control of anti-apoptotic factors expression would be critical for cells' ability to modulate its responses to apoptotic stimuli [71]. Thus, similarly to what we observed in pro-apoptotic factors, a reduction of anti-apoptotic proteins would be expected under resveratrol treatment.

Moreover, these results are in agreement with previous investigation showing an anti-apoptotic effect of resveratrol in pancreas [34] and heart [72,73].

Summarizing, resveratrol had positive effects on the heart of old animals even when starting supplementation at old age. Our results indicate that resveratrol might be an interesting approach not only for preventing age-related increases in inflammation and oxidative damage, but also for reverting those changes once they take place. In the current investigation we observed that the pro-inflammatory and pro-oxidative state that characterizes the aging process were reverted by resveratrol. Moreover, the age-related changes in apoptotic markers were also reverted by resveratrol treatment. In figure 5 we summarize some aspects of the pathways that have been investigated in the current study together with resveratrol effects through SIRT1. Similarly to previous studies

showing protective effects of resveratrol in cognitive performance [74] or endothelial dysfunction [75] in aged rats, our results suggest that resveratrol might be also used as anti-aging approach in order to revert the age-related changes in cardiac function.

ACKNOWLEDGMENTS. This research was supported by a grant from RETICEF (RETICEFRD12/0043/0032) to JAFT.

CONFLICT OF INTEREST. The authors declare that they have no conflicts of interest.

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690

691

FIGURE LEGENDS

Fig. 1 Sirtuin 1 protein levels in the heart of young (C 2m), old (C 24m) and old rats treated with resveratrol (RSV 24m). Results are expressed as the mean± SEM relative to 2 month-old animals.

Fig. 2 mRNA levels of inflammatory markers in the heart of young (C 2m), old (C 24m) and old rats treated with resveratrol (RSV 24m). Results are expressed as the mean± SEM relative to 2 month-old animals. n= 4- 6, except TNF-α data in C 24m group (n=3). *p≤0.05

Fig. 3 mRNA levels of oxidative markers in the heart of young (C 2m), old (C 24m) and old rats treated with resveratrol (RSV 24m). Results are expressed as the mean± SEM relative to 2 month-old animals. n= 4- 6, except eNOS and iNOS data in C 24m group (n=3). *p≤0.05; **p≤0.01; ***p≤0.001

Fig. 4 mRNA levels of apoptotic markers in the heart of young (C 2m), old (C 24m) and old rats treated with resveratrol (RSV 24m). Results are expressed as the mean± SEM relative to 2 month-old animals. n= 4-6, except XIAP and Bcl-2 data in C 24m group (n=3). *p≤0.05; **p≤0.01

Figure 5. Beneficial effects of resveratrol are partly related to sirtuin 1 actions. An important positive feedback between inflammation and oxidative stress takes place leading to activation of apoptotic events. Among other effects, resveratrol reduces inflammation and oxidative stress. The figure depicts some of the resveratrol effects through sirtuin 1. Those that have been analyzed in the current investigation are highlighted with boxes. Cyt c: Cytochrome c; C3:

714 Caspase 3; C8: Caspase 8; C9: Caspase 9; Solid lines with bars indicate

715 inhibition; Arrows represent activation.

716

717

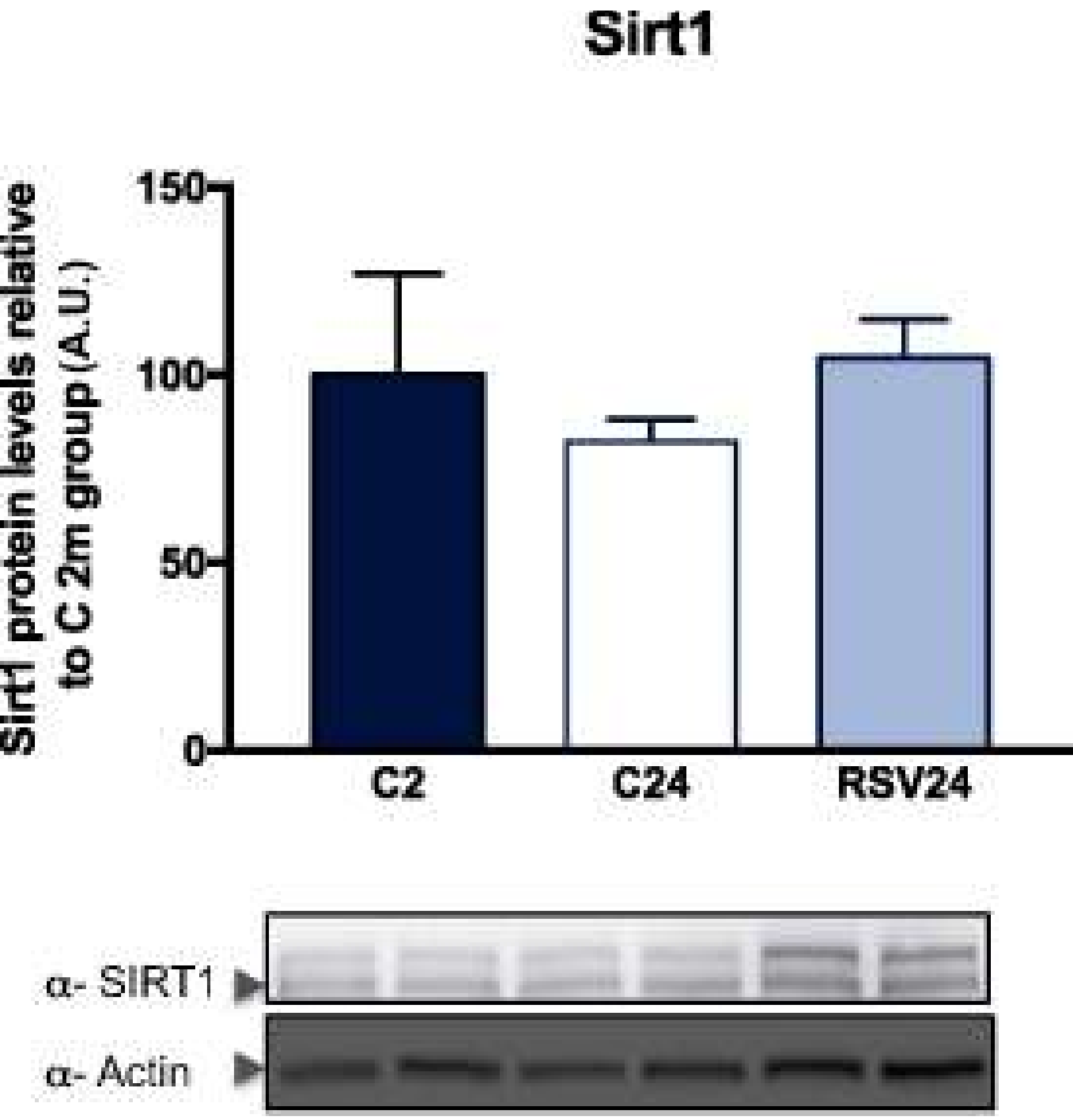
718

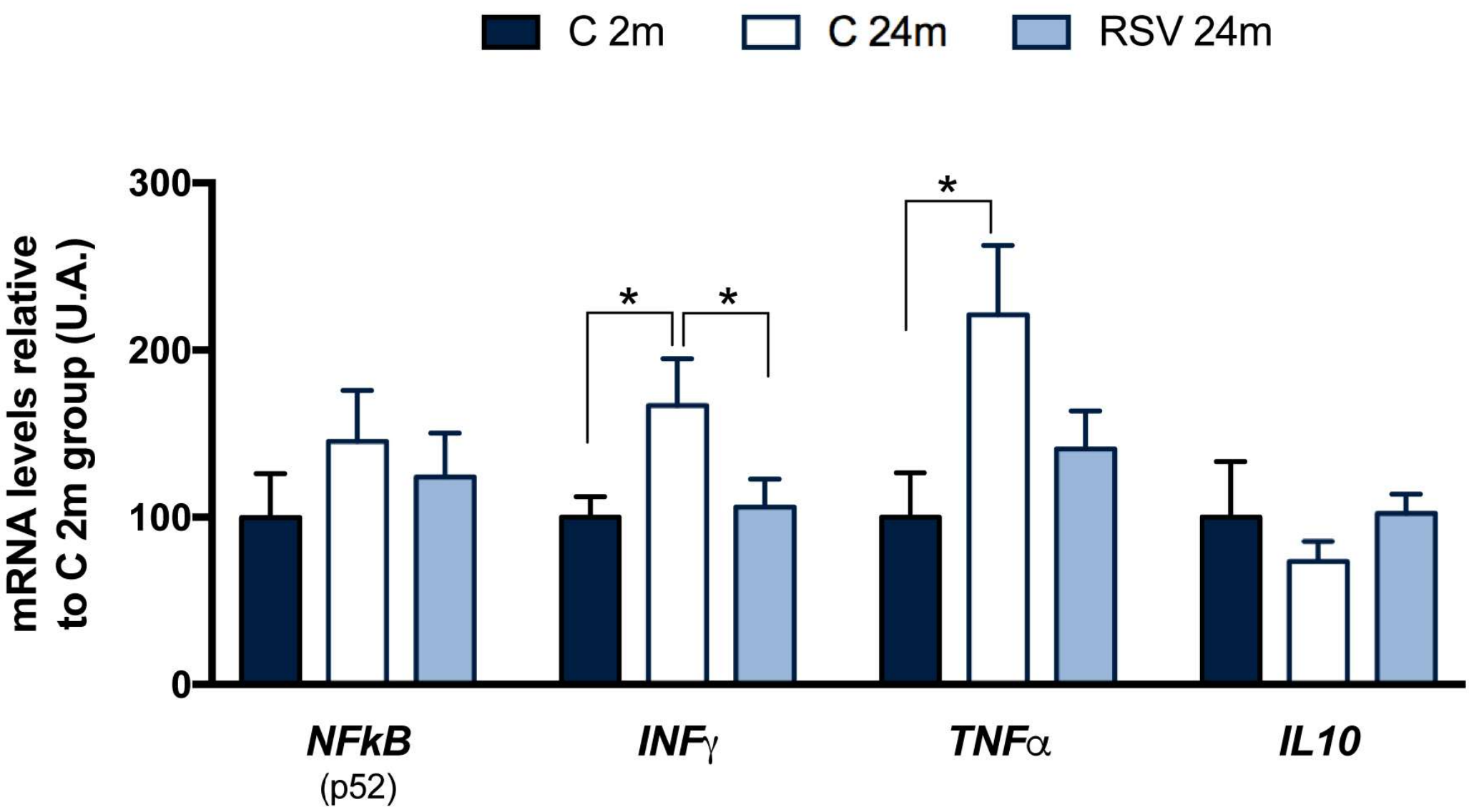
719 **Table 1. Primers used in RT-PCR experiments**

IL10	Forward	ACTGCACCCACTTCCCAGT
	Reverse	TTGTCCAGCTGGTCCTTTGT
NFkB	Forward	TGGAACAGCCCAAACAGC
	Reverse	CACCTGGCAAACCTCCAT
INFg	Forward	TGAAAGCCTAGAAAGTCTGAAGAAC
	Reverse	CGTGTTACCGTCCTTTTGC
TNF-a	Forward	ATGAGAAGTTCCCAAATGGC
	Reverse	CTCCACTTGGTGGTTTGCTA
AIF	Forward	AGTCCTTATTGTGGGCTTATCAAC
	Reverse	TTGGTCTTCTTTAATAGTCTTGTAGGC
XIAP	Forward	GCTTGCAAGAGCTGGATTTT
	Reverse	TGGCTTCCAATCCGTGAG
BAD	Forward	GCCCTAGGCTTGAGGAAGTC
	Reverse	CAAACTCTGGGATCTGGAACT
Bcl-2	Forward	CAGGTATGCACCCAGAGTGA
	Reverse	GTCTCTGAAGACGCTGCTCA
HO-1	Forward	GTCAAGCACAGGGTGACAGA
	Reverse	ATCACCTGCAGCTCCTCAAA
HO-2	Forward	TACGGCACCAGAAAAGGAAA
	Reverse	GTGCTTCCTTGGTCCCTTC
eNOS	Forward	CCAGTGCCCTGCTTCATC
	Reverse	GCAGGGCAAGTTAGGATCAG
iNOS	Forward	CTTTGCCACGGACGAGAC
	Reverse	TCATTGTACTCTGAGGGCTGAC
18S	Forward	GGTGCATGGCCGTTCTTA
	Reverse	TCGTTCGTTATCGGAATTAACC

720 18S was used as a housekeeping gene to compare the samples

Figure 1


Sirt1
Sirt1 protein levels relative to C 2m group (A.U.)
150
100
50
0
C2
C24
RSV24
α- SIRT1
α- Actin


C 2m
C 24m
RSV 24m
mRNA levels relative to C 2m group (U.A.)
300
200
100
0
*
*
*
NFkB
(p52)
INFγ
TNFα
IL10

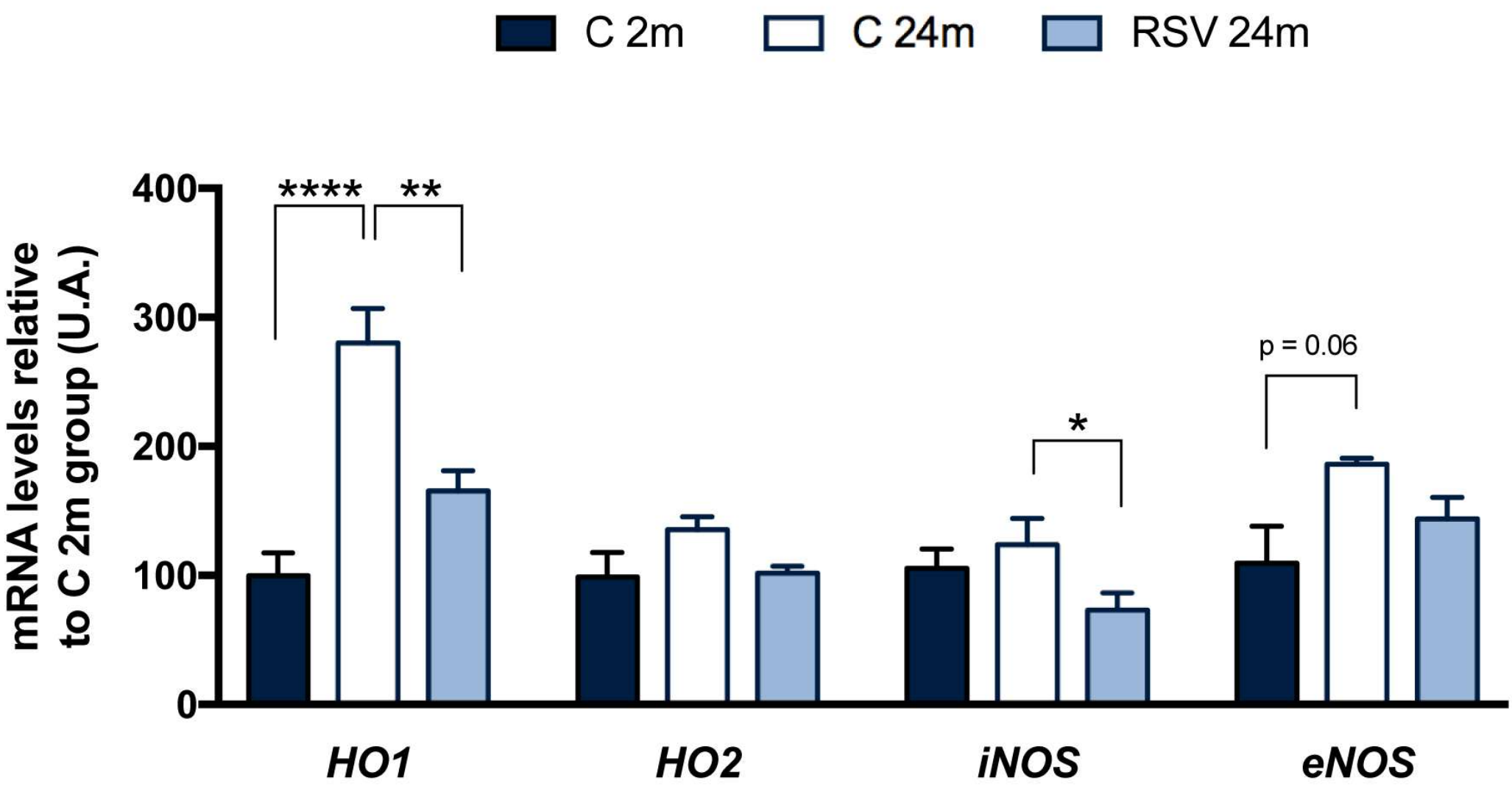

C 2m
C 24m
RSV 24m
mRNA levels relative to C 2m group (U.A.)
400
300
200
100
0
****
**
*
p = 0.06
HO1
HO2
iNOS
eNOS

Figure 4

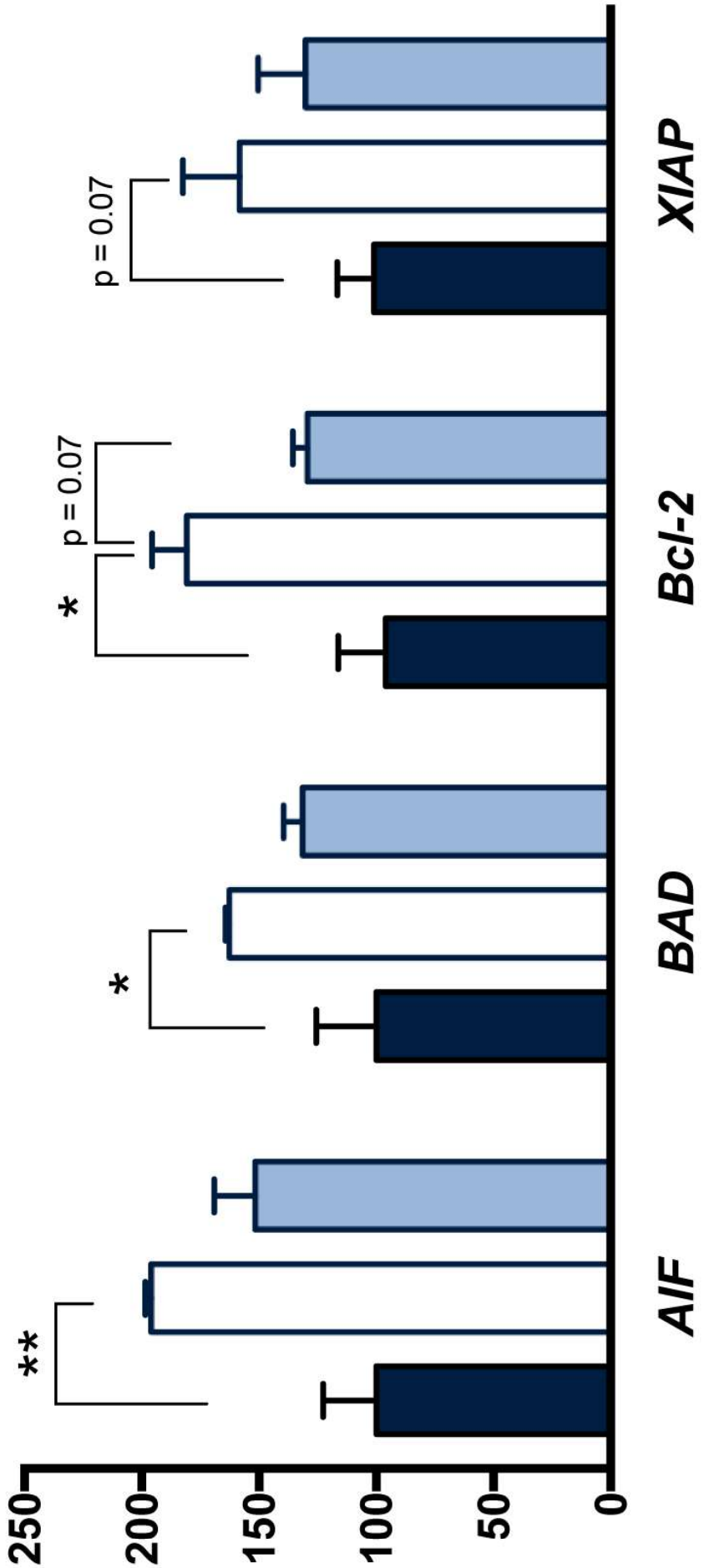
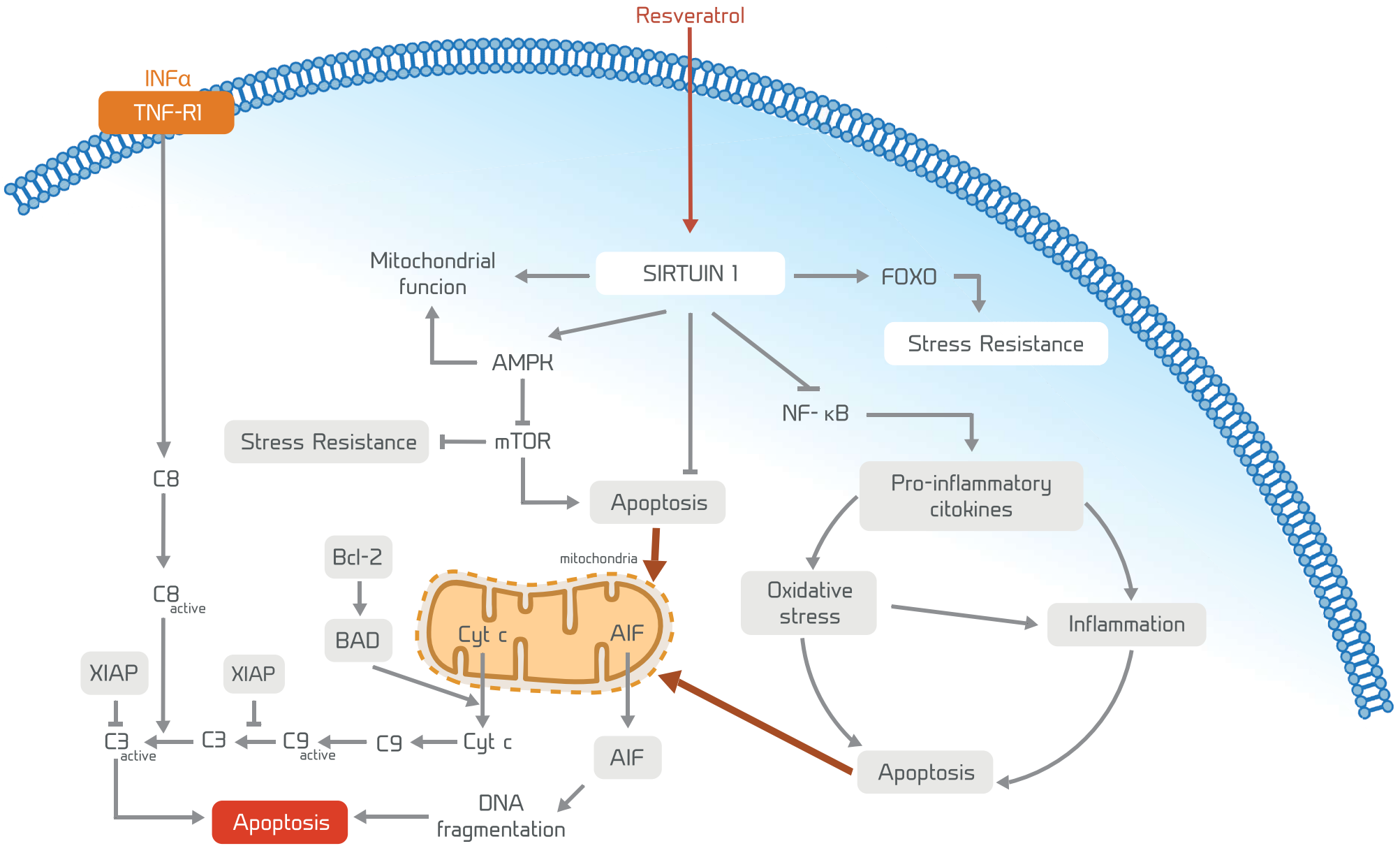

C 2m
C 24m
RSV 24m
mRNA levels relative to C 2m group (U.A.)
250
200
150
100
50
0
**
*
*
p = 0.07
p = 0.07
AIF
BAD
Bcl-2
XIAP

Figure 5


Resveratrol
INFα
TNF-R1
Mitochondrial funcion
SIRTUIN 1
FOXO
Stress Resistance
AMPK
NF- κB
Stress Resistance
mTOR
C8
Apoptosis
Pro-inflammatory citokines
Bcl-2
mitochondria
C8 active
Oxidative stress
Inflammation
BAD
Cyt c
AIF
XIAP
XIAP
C3 active
C3
C9 active
C9
Cyt c
AIF
Apoptosis
DNA fragmentation
Apoptosis