Reversal of doxorubicin-induced vascular dysfunction by resveratrol in rat thoracic aorta: Is there a possible role of nitric oxide synthase inhibition?

Sıçan torasik aortunda doksorubisinin oluşturduğu fonksiyon bozukluğunun resveratrol uygulaması ile düzelmesi: Nitrik oksit sentaz inhibisyonunun olası rolü var mı?

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ABSTRACT

Objective: The natural antioxidant, resveratrol has been suggested to protect against doxorubicin-induced cardiotoxicity. Although derangements in nitric oxide (NO) synthesis contribute to vascular endothelial dysfunction caused by doxorubicin, the effects of resveratrol on these parameters have not been evaluated yet. We investigated the impact of resveratrol on doxorubicin-induced vascular dysfunction in rat thoracic aorta with regard to NO synthesis in an experimental, prospective, controlled study.

Methods: Wistar rats were assigned to 5 groups; doxorubicin (n=9), vehicle (dimethylsulphoxide) (n=8), resveratrol (n=8), doxorubicin+resveratrol (n=10), controls (n=9). Contractile and relaxant responses were evaluated on the isolated thoracic aortas. The expressions of endothelial (eNOS) and inducible (iNOS) isoforms of NO-synthase were also examined histopathologically on the aortas. Statistical analysis was performed by ANOVA for repeated measures for the response curves and one-way ANOVA for the pD_2 (-log EC_{50}) and E_{max} (maximum phenylephrine contraction) values with subsequent Bonferroni test.

Results: Doxorubicin (20 mg/kg, i.p), not only decreased the contractile responses to phenylephrine ($p<0.001$), but also attenuated the relaxant responses to acetylcholine (ACh) ($p=0.002$), calcium ionophore (A23187) ($p=0.002$) and sodium nitroprusside (SNP) ($p=0.007$). Immunohistochemistry revealed increased ($p<0.05$) eNOS and iNOS protein expressions after doxorubicin treatment. Coadministration of resveratrol (10 mg/kg/i.p.) reversed the increased expression of both NOS isoforms ($p<0.05$). Similarly, it prevented the doxorubicin-induced attenuation in ACh- ($p=0.013$) and A23187- ($p=0.038$) induced responses. In healthy rats the antioxidant did not cause significant changes.

Conclusion: Prevention of excessive NO formation through eNOS and iNOS overexpression by resveratrol might contribute to the reversal of vascular endothelial dysfunction associated with doxorubicin treatment. *(Anadolu Kardiyol Derg 2009; 9: 260-6)*

Key words: Doxorubicin, vascular endothelial function, nitric oxide, resveratrol, rat

Özet

Amaç: Doğal bir antioksidan olan resveratrolün doksorubisine bağlı kardiyotoksisiteye karşı koruduğu öne sürülmektedir. Nitrik oksit (NO) sentezindeki düzensizliklerin doksorubisine bağlı damar endotel disfonksiyonuna katkısı olduğu düşünülmekle birlikte, resveratrolün söz konusu parametreler üzerindeki etkileri henüz araştırılmamıştır. Deneysel, prospektif, kontrollü çalışmada resveratrol uygulamasının sıçan torasik aortunda doksorubisinin oluşturduğu endotel fonksiyon değişiklikleri üzerine etkileri ve bu etkilerin NO sentezi ile ilişkisi incelenmiştir.

Yöntemler: Wistar sıçanları 5 gruba ayrıldı; doksorubisin (n=9), çözücü (dimetilsülfoksit) (n=8), resveratrol (n=8), doksorubisin+resveratrol (n=10), kontrol (n=9). Gruplardan izole edilen torasik aort preparatlarında kasılma ve endotel aracılı/aracısız gevşeme yanıtları ile değişik NO-sentaz (NOS) izoformlarının ekspresyon düzeyleri histopatolojik olarak değerlendirildi. Doz-yanıt eğrilerinin istatistiksel değerlendirmesinde tekrarlayan ölçümler için ANOVA, eğrilerin pD_2 ve E_{max} (maksimum fenilefrin kontraksiyonu) değerleri için ise tek yönlü ANOVA yöntemlerinin ardından Bonferroni testi uygulandı.

Bulgular: Doksorubisin uygulaması (20 mg/kg, i.p.), endotelli preparatlarda fenilefrinle indüklenen kontraktil yanıtların ($p<0.001$) yanı sıra, asetilkolin (ACh) ($p=0.002$), kalsiyum iyonofor (A23187) ($p=0.002$) ve sodyum nitroprusit (SNP) ($p=0.007$) ile elde edilen gevşeme yanıtlarında

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azalmaya neden oldu. İmmünohistokimyasal incelemelerde doksorubisin uygulamasından sonra endotelyal NOS (eNOS) ve indüklenebilir NOS (iNOS) ekspresyon düzeylerinin anlamlı olarak arttığı gözlendi ($p<0.05$). Sadece resveratrol (10 mg/kg/i.p.) uygulanan grupta eNOS ya da iNOS ekspresyonu değişmedi. Ancak doksorubisin+ resveratrol uygulanan grupta NOS enzim ekspresyon düzeyi doksorubisin grubuna göre azaldı ($p<0.05$). Resveratrol tek başına damar yanıtlarında belirgin değişiklikler oluşturmazken, birlikte uygulandığında doksorubisin tarafından azaltılan ACh ($p=0.013$) ve A23187 ($p=0.038$) yanıtlarında anlamlı artışlara neden oldu.

Sonuç: Endotelyal ve indüklenebilir NOS izoformlarının ekspresyonunda artışa bağlı aşırı NO üretiminin resveratrol tarafından önlenmesi doksorubisin tedavisi ile ilişkili damar endotel fonksiyon bozukluğunun düzelmesine katkıda bulunabilir. *(Anadolu Kardiyol Derg 2009; 9: 260-6)*

Anahtar kelimeler: Doksorubisin, damar endotel fonksiyonu, nitrik oksit, resveratrol, sıçan

Introduction

The widely used antineoplastic drug, doxorubicin produces a dose-related cardiomyopathy as a serious side effect, which makes its clinical utility limited (1, 2). Previous studies have shown that besides its toxic effect on cardiomyocytes, doxorubicin also causes endothelial damage, which contributes to its cardiotoxicity and other side effects (3, 5). The production of reactive oxygen species as well as derangements in nitric oxide (NO) synthesis are considered as the possible mechanisms by which doxorubicin causes endothelial dysfunction. The possible mechanisms by which doxorubicin causes endothelial dysfunction include the production of reactive oxygen species and derangements in nitric oxide synthesis (6, 8). This semiquinone has been shown to bind to the reductase domain of endothelial nitric oxide synthase (eNOS), resulting in the diversion of electron flow from the oxygenase domain of the enzyme, thus leading to an increase in superoxide generation and reduction in NO production (6). Since vascular endothelium plays a fundamental role in maintenance of organ function several approaches have been tried to ameliorate the vascular toxicity of this agent .

Resveratrol, a natural phytoalexin produced in grapes, is present in wine together with other polyphenols and has the potency to scavenge peroxyl radicals (9). Although it is not a very potent antioxidant *in vitro*, it functions as a potent antioxidant *in vivo* and has been shown to exert cardioprotective effects (10, 11). This effect is believed to be at least partially due to resveratrol's antioxidant activity, but its potential role as a pharmacological preconditioning agent acting through NO synthesis has also been considered (12). Concomitant administration of resveratrol with doxorubicin has recently been suggested as a novel strategy to protect against the cardiotoxicity doxorubicin of and was demonstrated to increase the viability of cardiac myocytes *in vitro* (13, 14). However, although vascular endothelial dysfunction and derangements in NO synthesis are common features of doxorubicin toxicity, the potential beneficial effects of doxorubicin-resveratrol combination on these parameters have not been evaluated yet. Thus, we aimed to investigate the effects of resveratrol on doxorubicin-induced changes on vascular endothelial function in the rat aorta with regard to NO synthesis using a cardiotoxic dose (15) of the antineoplastic agent.

Methods

The project was approved by the Local Animal Care and Ethics Committee. The instructions and policies of this committee conform with the Guide for the Care and Use of Laboratory Animals Published by the US National Institutes of Health (NIH Publication No: 85-23, revised 1996).

Drugs and reagents

Resveratrol was provided from Cayman Chemical Company (Ann Arbor, USA). Doxorubicin was the commercially available form used in Turkey (Adriablastina flacon, DEVA Company). The following drugs were purchased from Sigma (USA): DMSO, phenylephrine, ACh, SNP, A23187. For vascular reactivity studies, stock solutions of drugs were prepared in distilled water and the concentrations of the drugs were expressed as final molar concentrations in the bath solution.

Study protocol

Fifty male Wistar rats (200-250 gr) were used in this prospective, controlled study. All animals were fed *ad libitum* with standard plain diet and were allowed free access to water. The day/night cycle was 12:12 hours and room temperature was maintained at 21± 3°C.

Rats were randomly assigned to 5 groups of 10 animals each; Group 1 (Doxorubicin): The rats in this group received a single intraperitoneal (i.p.) injection of doxorubicin (20 mg/kg /b.w.) 7 days prior to functional studies. Group 2 (Vehicle): These rats received a single i.p. injection of the vehicle (dimethylsulphoxide-DMSO) at equivalent amounts with the resveratrol-treated rats. Group 3 (Resveratrol): This group received a single i.p. injection of resveratrol (10 mg/kg/b.w.). Group 4 (Doxorubicin + Resveratrol): The rats in this group were given doxorubicin and resveratrol combination intraperitoneally 1 week before sacrifice. Group 5 (Controls): These rats which served as the control group, received a single dose of saline injection.

At the end of the study period, rats were sacrificed by withdrawal of blood by cardiac puncture under ketamine (70 mg/kg) and xylazine (10 mg/kg) anesthesia.

Thoracic aortas from a total of 6 rats were not used in the studies because of the unexpected experimental conditions which were thought to effect the safety of the data.

Vascular reactivity studies

After sacrifice, thoracic aortas were quickly removed into 4°C Krebs-Henseleit solution. After removal of fat the whole aorta was cut into approximately 5 mm rings. For a given experiment, the aorta rings with endothelium were suspended horizontally in a 20 ml organ bath containing Krebs solution (composition in mM: NaCl 118.1, KCl 4.7, $CaCl_2$ 2.5; $MgSO_4$ 1.2,

$NaHCO_3$ 25.0, KH_2PO_4 1.22, glucose 11.1; pH= 7.4) which was maintained at 37°C and continuously gassed with a mixture of 95% oxygen and 5% carbon dioxide under an initial tension of 2 g. Tension was recorded isometrically with a force transducer (COMMAT, Ankara, Turkey) and displayed on a Biopac acquisition system (Biopac System Inc., CA, U.S.A.).

After 15 minutes (min) of equilibration, each ring was systematically stretched to the optimum of its length–active tension by exposure to incremental concentrations of potassium chloride (KCl). Rings were then left to equilibrate in the bath for a total of 30 min. and washed every 15 min. After the initial equilibration period of 60-90 min endothelium-intact rings were used to assess the contractile responses elicited by incremental concentrations of phenylephrine (0.001-30 μM). Relaxant responses were also determined using cumulative concentrations of acetylcholine (ACh) (0.001-30 μM), calcium ionophore (A23187) (0.001-3 μM) or sodium nitroprusside (SNP) (0.0001-0.3 μM) on endothelium-intact rings precontracted with submaximal concentration of phenylephrine. In order to maintain appropriate precontractile tension in all preparations, submaximal concentrations were determined using the prior cumulative concentration-response curves of phenylephrine.

Tissue processing and immunohistochemistry

Tissue pieces from thoracic aortas were fixed in 4% paraformaldehyde (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) for 24 hours (h), at 4°C and processed for embedding in paraffin wax using routine protocols. Sections were cut using a microtome (Leica MR 2145); and were dewaxed and rehydrated through a graded ethanol series. Sections were then washed with distilled water and phosphate buffered saline (PBS) for 10 min and treated with 2% trypsin (Sigma Chemical Co. St. Lois, Missouri, USA) in 50 mM Tris buffer (pH 7.5), at 37°C, for 15 min. Sections were then delineated with a Dako pen (Dako, Glostrup, Denmark). Blocking of endogenous peroxidase activity was achieved using hydrogene peroxide (3%) in absolute alcohol for 15 min. Sections were incubated with primary antibodies directed against iNOS (1:100 dilution; Abcam, Cambridge, UK) and eNOS (1:1000 dilution; Abcam, Cambridge, UK) all for 18 h at 4°C in a humid chamber. After incubation with biotinylated secondary antibody and streptavidin conjugated to horseradish peroxidase (both from Zymed Histostain-plus-Peroxidase-kit, 85-9043, San Francisco, CA, USA) for 30 min each respectively, sections were finally incubated with diaminobenzidine (DAB) (from DeadEnd Colorimetric TUNEL system, Promega, Madison, USA) for 5 min to reveal immunolabelling. All dilutions and thorough washes between stages were performed using PBS. Sections were counterstained with Mayer's hematoxylin (Zymed Laboratories, U.S.A.). After washing with tap water, sections were dehydrated through a graded ethanol series, cleared in xylene and mounted with entellan (Merck). Negative control samples were processed as described above except that primary antibodies were omitted and replaced with PBS alone. Positive controls were represented by sections of a neuroblastoma specimen known to be positive for the markers of interest.

Evaluation of sections

Immunoreactivities of iNOS and eNOS were evaluated in the vascular tissue achieved from all experimental groups. Endothelial NOS activity was particularly investigated in the endothelium. Immunohistochemistry were evaluated semiquantitatively (Olympus BX-51 and Olympus C-5050 digital camera) using an additive immunoreactive score reflecting signal intensity, that is 0-negative, 1-weak, 2-intermediate and 3-strong, and the number of immunopositive cells, that is 0-no positive cells, 1-less than 10% positive cells, 2-10% to 50% and 3-greater than 50%. The 2 scores were added. Measurements were performed by two independent researchers blind to the groups.

Statistical analyses

Statistical analysis was performed with SPSS (SPSS Inc., Chicago, IL, USA) system for Windows, version 13.0. Results are expressed as mean±SD of the groups except for the concentration-response curves which the data were presented as mean ± S.E.M. Concentration-response curves were fitted by non-linear regression with simplex algorithm and pD_2 (-log EC 50) values were calculated using the software of transducer data acquisition system (Polywin 95 Ver. 1.0. COMMAT İletişim Ltd., Ankara, Turkey). Relaxant responses are given as the percentage changes from phenylephrine precontraction. Comparisons of concentration-response curves were evaluated by two-way analysis of variance (ANOVA) for repeated measures followed by Bonferroni test. The differences between the means of pD_2 and E_{max} (Maximum contraction to phenylephrine) values for the concentration-response curves were assessed by one-way ANOVA with a subsequent Bonferroni test. A p value <0.05 was considered statistically significant.

Results

Vascular reactivity studies

In the thoracic aortas, doxorubicin significantly decreased the contractile responses to phenylephrine, when compared to the control or vehicle-treated groups ($p<0.001$ and $p<0.001$ respectively) (Fig. 1), without a significant change in pD_2 values (Table 1). Maximum responses to phenylephrine (E_{max}) were also decreased in these rings ($p<0.001$), (Table 1). Contractile

Table 1. The E_{max} and pD_2 values of phenylephrine in endothelium-intact rings

Groups	pD_2	E_{max}*
Control, n=9	7.34±0.46	1430.57±327.08
Vehicle, n=8	6.96±0.45	1437.53±243.66
Doxorubicin, n=9	7.07±0.67	653.37±136.9[a]
Doxorubicin+Resveratrol, n=10	7.13±0.39	890.00±272.81[a]
Resveratrol, n=8	7.27±0.26	1631.94±99.13

Data are presented as mean ±S.D.
*One-way ANOVA analysis, $p<0.001$, $F(4.39)= 31.01$
[a]:posthoc Bonferroni test; $p< 0.001$, compared to the control group

responses tended to increase with concomitant administration of resveratrol to doxorubicin-treated rats, but the increase was not statistically significant. Rings from resveratrol-treated rats showed no significant differences regarding phenylephrine responses, when compared to control or vehicle-treated groups.

Endothelium-intact rings precontracted with phenylephrine gave a concentration-dependent relaxant response to ACh, A23187 and SNP (Fig. 2-4, Table 2). Acetylcholine-induced relaxation responses were found to be decreased in the doxorubicin-treated preparations when compared to the control group (p=0.002), but no changes were observed among the pD_2 values. Administration of resveratrol with doxorubicin significantly reversed the decrease in the relaxation induced by ACh (p=0.013), whereas resveratrol alone did not cause significant differences when compared to the control and vehicle groups in terms of endothelium-dependent relaxation (Fig. 2).

The responses to A23187 significantly decreased in aortas from doxorubicin-treated rats compared to the control (p=0.002) or vehicle group (p=0.024) (Fig. 3). Resveratrol, when given with doxorubicin significantly increased relaxation compared to the group given doxorubicin alone (p=0.038) . pD_2 values for the response curves did not differ among the experimental groups (Table 2).

Endothelium-independent relaxations induced by SNP were attenuated in the aortas from doxorubicin-treated rats (p=0.007, Fig. 4), with significantly decreased pD_2 values (p<0.005, Table 2). Concomitant administration of resveratrol with doxorubicin did not cause any difference in SNP-induced relaxation response, but significantly increased the pD_2 values of the response

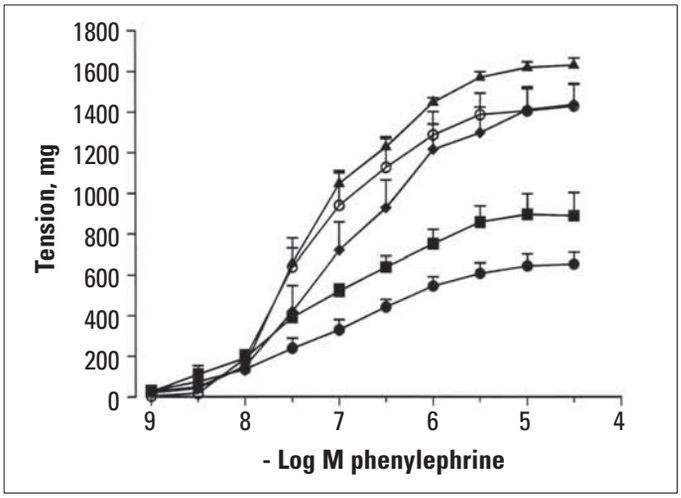

Figure 1. Concentration response curves of phenylephrine in endothelium-intact thoracic aortic rings

Data are expressed as means ± S.E.M. of the groups; n=8-10 in each group (○-control, ♦-vehicle, ●-doxorubicin, ▲-resveratrol, ■-doxorubicin+resveratrol). Doxorubicin significantly decreased the contractile responses to phenylephrine, when compared to the control group. ANOVA for repeated measures ; p<0.001, F (4.39)= 22.79; posthoc Bonferroni test: Doxorubicin vs control (p<0.001)

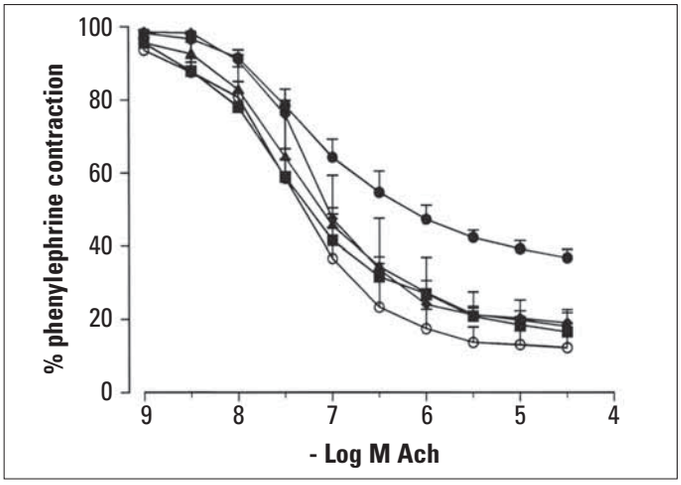

Figure 2. Concentration-response curves of acetylcholine (ACh) in endothelium intact thoracic aortic rings precontracted with a submaximal concentration of phenylephrine

Data are expressed as means ± S.E.M. of the groups; n=8-10 in each group (○-control, ♦-vehicle, ●-doxorubicin, ▲-resveratrol, ■-doxorubicin+resveratrol). Administration of resveratrol with doxorubicin significantly reversed the decrease in the relaxations induced by ACh. ANOVA for repeated measures ; p<0.001, F (4.39)= 6.55; posthoc Bonferroni test: Doxorubicin vs control (p=0.002), doxorubicin vs doxorubicin+resveratrol (p=0.013)

Table 2. The pD_2 values of the concentration-response curves obtained by different relaxant agents

Groups	Acetylcholine	A-23187	SNP*
Control, n=9	7.73±0.37	6.86±0.79	7.87±0.36
Vehicle, n=8	7.39±0.36	6.84±0.49	7.81±0.28
Doxorubicin, n=9	7.52±0.42	6.43±0.61	6.86±0.27[a]
Doxorubicin+Resveratrol, n=10	7.38±0.52	6.46±0.58	7.65±0.41[b]
Resveratrol, n=8	7.13±0.72	6.16±0.50	8.24±0.31

Data are presented as mean ± S.D.
*One-way ANOVA analysis, p<0.001, F (4,39)= 20.17
[a, b]posthoc Bonferroni test;
[a]p<0.001 compared to the control group
[b]p=0.001 compared to the doxorubicin-treated group

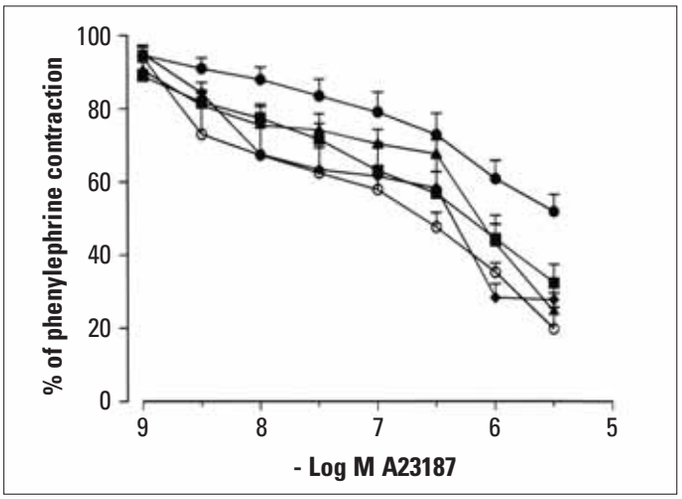

Figure 3. Concentration-response curves of calcium ionophore (A23187) in endothelium intact thoracic aortic rings precontracted with a submaximal concentration of phenylephrine

Data are means ± S.E.M. of the groups; n= 8-10 in each group (○-control, ♦-vehicle, ●-doxorubicin, ▲-resveratrol, ■-doxorubicin+resveratrol). Concomitant administration of resveratrol with doxorubicin significantly increased the relaxations when compared to the group treated with doxorubicin alone. ANOVA for repeated measures; p<0.001, F (4,39)=5.54; posthoc Bonferroni test: Doxorubicin vs control (p=0.002), doxorubicin vs doxorubicin+resveratrol (p=0.038)

curves (p=0.001, Table 2), revealing increased sensitivity of the aortas to SNP. Comparison of the responses from the group treated with resveratrol alone showed no significant changes when compared to the control or vehicle groups.

iNOS and eNOS immunoreactivity in aortic sections

Histological analysis of the aortas demonstrated that both iNOS and eNOS immunoreactivities were significantly increased in the doxorubicin group (Fig. 5B) compared to the control aortas (Fig. 5A). Marked staining of iNOS was observed in the slices from the aortas of doxorubicin-treated rats and the activity was more pronounced especially in tunica media. Inducible NOS and eNOS immunoreactivities were decreased when resveratrol was given concomitantly with doxorubicin (Fig. 5C). Resveratrol (Fig. 5D), or vehicle (not shown) administration alone revealed similar immunoreactive effects compared to the control group in all vessel compartments.

Results of the semiquantitative analysis revealed that immunoreactive scores for both iNOS and eNOS were increased in the doxorubicin group (p<0.05), whereas marked decrease was observed after doxorubicin and resveratrol combination therapy (p<0.05) (Fig 6). Resveratrol alone did not cause any changes in terms of immunoreactivity when compared to the control group.

Discussion

The data obtained in the *in vivo* experiments showed that a single cardiotoxic dose of doxorubicin not only decreased the contractile responses to phenylephrine in endothelium-intact thoracic aortas, but also attenuated the relaxant responses to ACh, A23187 and SNP. Immunohistochemistry revealed significantly increased protein expression levels of eNOS and iNOS after doxorubicin treatment.

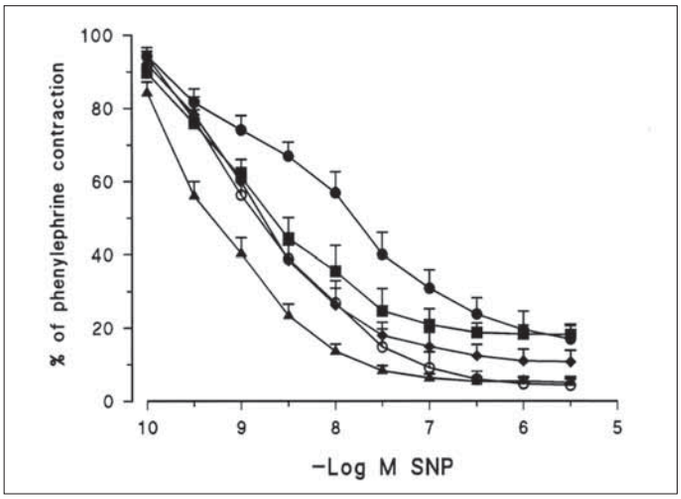

Figure 4. Concentration-response curves of sodium nitroprusside in endothelium intact thoracic aortic rings precontracted with a sub-maximal concentration of phenylephrine

Data are means ± S.E.M. of the groups; n= 8-10 in each group (○-control, ♦-vehicle, ●-doxorubicin, ▲-resveratrol, ■-doxorubicin+resveratrol). Coadministration of resveratrol did not reverse the decreased SNP-induced relaxations in doxorubicin treated group. ANOVA for repeated measures; p<0.001, F (4,39)= 12.62; posthoc Bonferroni test: Doxorubicin vs control (p=0.007), doxorubicin vs doxorubicin+resveratrol (p=0.15, N.S)

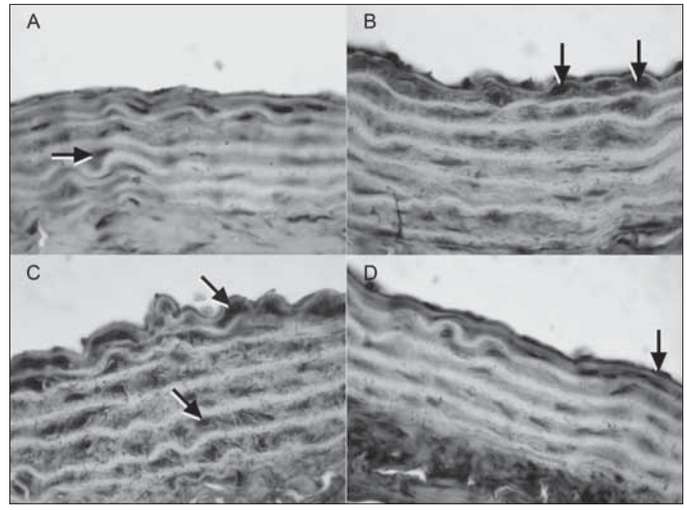

Figure 5. Immunoreactivity of nitric oxide synthase isoforms demonstrated by immunohistochemistry in rat thoracic aortas (n=8-10). Arrows show immunohistochemically reactive cells in different vessel compartments (A) Control, (B) Doxorubicin, (C) Doxorubicin+resveratrol, (D) Resveratrol. Evaluation of the slices from the doxorubicin group revealed increased immunoreactivities of both NOS isoforms. Figure 5B demonstrates marked staining of iNOS in tunica media from the aorta of a doxorubicin-treated rat. Immunoreactivity was decreased when resveratrol was given concomitantly with doxorubicin as represented for iNOS in Figure 5D

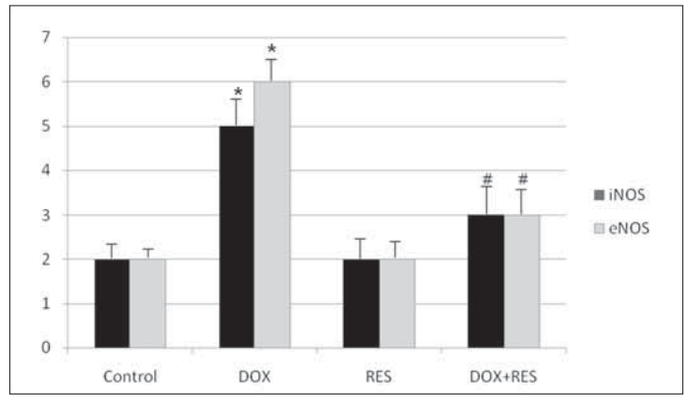

Figure 6. Results of the semiquantitative analysis of iNOS and eNOS immunoreactivity in rat thoracic aortas (n=8-10). Immunoreactive scores for both iNOS and eNOS were increased (p<0.05) in the doxorubicin group when compared to the controls. Significant decrease was observed after doxorubicin and resveratrol combination therapy (p< 0.05)

*: Significantly different when compared to the control group.

#: Significantly different when compared to the doxorubicin-treated group

These data are in agreement with the findings of a previous study, which reported attenuation of ACh and A23187- induced responses by a single dose of doxorubicin (10 mg/kg, b.w., i.v.) in rabbit aortas (7). These authors also demonstrated that the vasorelaxation defect in the aortas was accompanied by endothelial generation of oxygen radicals and reduced plasma NO levels. In fact, it has been supposed that doxorubicin could rapidly generate superoxide radicals from the reductase domain of fully coupled eNOS in a dose dependent fashion resulting in the diversion of electron flow from the oxygenase domain of the enzyme, thus leading to an increase in superoxide generation and a concomitant reduction in NO production (6). It is well

known that NO release by ACh is facilitated by muscarinic receptors, while A23187 works in a receptor-independent manner. Both agents increase intracellular Ca^{++} in the endothelial cells, which in turn activates eNOS (16). Thus, the decreased responses to both ACh and A23187 in our study is more likely due to a possible defect in eNOS activity, rather than an abnormality in ACh receptor/signal transduction. This finding also supports the hypothesis that although eNOS activity is increased by doxorubicin, the decrease in the formation of NO with concomitant increase in oxygen radicals might be responsible of the generalized reduction in relaxant responses.

On the other hand, the attenuation of SNP responses by doxorubicin has also been reported in rabbit aorta by Duquaine and colleagues and has been attributed to the proapoptotic effect of the agent (7). It is known that tissue catalyzed reduction of SNP results in NO production in an endothelium-independent manner and directly activates the cytosolic fraction of vascular smooth muscle guanylyl cyclase causing intracellular increase of the endogenous vasodilator cGMP. Thus, the decrease in SNP-induced responses suggests that doxorubicin not only decreases the bioavailability of NO, but possibly causes a defect in the guanylyl cyclase-cGMP pathway or a direct toxic effect on the smooth muscle cells as well.

Since doxorubicin causes a generalized relaxant attenuation in doxorubicin-treated aortas, one could easily expect the phenylephrine-induced contractile responses to be increased. It is known that in rat aortas spontaneous release of NO is a functional antagonist of the contractions induced by alpha-adrenergic agonists and the release of basal endothelium-derived NO (17). Thus, the impaired NO production by doxorubicin would be expected to lead to increased phenylephrine responses. However, contrary to this hypothesis we found significantly decreased contractile responses to phenylephrine in doxorubicin-treated rat aortas. In fact, the results of a previous study by Murata et al. provide a plausible explanation for this finding (3). These authors demonstrated that doxorubicin inhibited noradrenaline-induced contractions in rabbit mesenteric arteries *in vitro* and this effect was found to be due to the decreased expression of the $\alpha 1_A$-adrenoceptors. Since noradrenaline-induced contractions were restored by superoxide dismutase administration superoxide radical anions were thought to be responsible for the down regulation of the receptors at the protein synthesis step regardless of NO synthesis.

It has recently been shown that resveratrol or its oligomers had protective effects against doxorubicin-induced oxidative injury on rat cardiac cell cultures, and human gynecologic cancer cell lines (13, 14). This naturally occurring polyphenol is a potent antioxidant *in vivo* and has also been demonstrated to inhibit iNOS expression and iNOS enzymatic activity in different cell lines (18, 19). However, discrepancy exists on the effect of resveratrol on eNOS expression. Endothelial cell culture studies have revealed that resveratrol increased both eNOS activity and expression, whereas in a recent study chronic *in vivo* administration of the polyphenol did not alter eNOS levels in rat aortas, despite significant enhancement of endothelium-dependent relaxation (20-22). Using immunohistochemistry, we demonstrated that resveratrol alone did not effect eNOS or iNOS expression when compared to control aortas, but decreased the expression of both enzymes, which were increased by doxorubicin. Similarly, it did not cause significant changes when compared to control aortas in terms of vascular reactivity studies. However, when administered concomitantly with doxorubicin it reversed the attenuated responses to ACh and A23187, which were decreased by doxorubicin and increased the sensitivity of the aortas to SNP-induced relaxations.

Taken together, it might be speculated that resveratrol does not effect eNOS expression in healthy rat aortas, but reverses doxorubicin-induced eNOS activity, possibly through a decrease in free radical formation. The decrease in free radical formation prevents endothelial damage induced by doxorubicin and restores endothelium-dependent ACh-induced relaxation. Prevention of the increased eNOS activity that results in the diversion of electron flow from the oxygenase domain of the enzyme possibly leads to decreased free radical generation with a concomitant increase in NO production and restores the decreased responses to A23187. This assumption is in association with the findings of a recent study, which reported that disruption of eNOS protected against the cardiac injury, dysfunction, and mortality induced by doxorubicin (23). On the other hand, since induced iNOS expression by doxorubicin has also been shown to play a role in doxorubicin-induced cardiac and vascular dysfunction, it is noteworthy that inhibition of this NOS isoform by resveratrol might also contribute to the reversal of vascular function (24, 25).

On the other hand, the down-regulation of $\alpha 1_A$-adrenoceptors by excessive superoxide formation is supposed to be responsible of the attenuated phenylephrine responses by doxorubicin and our finding that the decreased contractile responses to phenylephrine were partially, but not significantly reversed by resveratrol indirectly supports the hypothesis that the direct antioxidant effect of resveratrol, regardless of its effect on NO synthesis might also contribute to the reversal of the doxorubicin-induced vascular dysfunction.

Limitations of the study

Although discussed with the findings of previous studies on the issue, our results from the present experiments do not provide direct evidence for the involvement of the antioxidant effect of resveratrol on doxorubicin-induced endothelial dysfunction regarding NO synthesis .

Conclusion

Data from our study consider that prevention of excessive NO formation through NOS overexpression by resveratrol may contribute to the restoration of vascular endothelial dysfunction which is associated with doxorubicin treatment. However, since this is the first study investigating the effects of resveratrol on

doxorubicin-induced vascular dysfunction, further studies will be of benefit to reveal the possible mechanisms underlying these findings.

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