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Doxorubicin-induced expression of LOX-1 in H9c2 cardiac muscle cells and its role in apoptosis

Paolo Spallarossa [a,1], Patrizia Fabbi [a,*,1], Valeria Manca [a], Silvano Garibaldi [a], Giorgio Ghigliotti [a], Chiara Barisione [a], Paola Altieri [a], Franco Patrone [b], Claudio Brunelli [a], Antonio Barsotti [a]

[a] *Research Center of Cardiovascular Biology, Department of Cardiology, University of Genova, Italy*
[b] *Department of Internal Medicine, University of Genova, Italy*

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Abstract

Up-regulation of LOX-1 is implicated in apoptosis in both vascular smooth muscle cells and in endothelial cells. We examined the effects of doxorubicin on LOX-1 expression in H9c2 cardiomyocytes and the role played by LOX-1 up-regulation in doxorubicin-induced apoptosis. Reactive oxygen species (ROS) formation was assessed by DCF flow cytometry. LOX-1 mRNA and protein expression was assessed by RT-PCR and Western blotting. Apoptosis was evaluated by flow cytometry with annexin/PI double staining. Doxorubicin-induced LOX-1 expression in a concentration- and time-dependent fashion. The doxorubicin-induced ROS formation and the LOX-1 expression were significantly attenuated by pre-treatment with antioxidants. By exposing cells that had been pre-treated with doxorubicin to oxidized-LDL, a LOX-1 agonist, in the presence or in the absence of k-carrageenan, a LOX-1 receptor antagonist, we documented that doxorubicin-induced LOX-1 expression plays a role in inducing apoptosis. These findings suggest that LOX-1 up-regulation is redox-sensitive and may contribute to doxorubicin-induced cardiotoxicity.

Keywords: Antioxidant; Apoptosis; Cardiomyocytes; Cardiotoxicity; Doxorubicin; LOX-1 receptor; Oxygen radicals

Lectin-like oxidized LDL receptor-1 (LOX-1) is a cell surface receptor for oxidized LDL (ox-LDL) and is predominantly expressed in activated endothelial and vascular smooth muscle cells, macrophages, and platelets [1–4]. LOX-1 plays a crucial pathogenetic role in atherosclerosis, in that it up-regulates the expression of various adhesion molecules, reduces nitric oxide availability, induces ROS formation, and contributes to plaque destabilization and thrombotic complications by facilitating the uptake of ox-LDL [5].

Recent studies have shown that LOX-1 is also expressed in the cardiac myocytes of failing hearts, but not in the cardiac myocytes of normal hearts, and that LOX-1 cardiomyocyte expression is up-regulated by stimulation with norepinephrine and endothelin, or following ischemia–reperfusion [6,7]. In addition, LOX-1 expression appeared to be associated with cardiac myocyte apoptosis and left ventricular functional deterioration [6,7].

Doxorubicin is one of the most effective antitumor agents, but its clinical use is limited by cumulative dose-related cardiotoxicity, which may lead to severe and irreversible cardiomyopathy [8]. The cause of doxorubicin cardiotoxicity is multifactorial, even though most doxorubicin-induced cardiac effects can be attributed to the formation of reactive oxygen species (ROS), which ultimately results in myocyte apoptosis [9].

* Corresponding author. Fax: +390103538655.
E-mail address: biopatty@yahoo.com (P. Fabbi).
[1] Both authors contributed equally to this study.

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In our study, we investigated the effects of doxorubicin on the expression of LOX-1, and the role LOX-1 up-regulation plays in doxorubicin-induced apoptosis in H9c2 cardiac muscle cells.

Materials and methods

Materials. Fetal bovine serum, phosphate-buffered saline, and cell culture medium were purchased from Euroclone (Wetherby, UK). Bicinchoninic acid (BCA) protein assay was bought from Pierce (Rockford, IL, USA). The cardioprotective agents we tested were: carvedilol (a gift from Roche, Monza, Italy), atenolol (AstraZeneca, Milan, Italy), dexrazoxane (Chiron, Amsterdam, The Netherlands), and *N*-acetylcysteine (NAC) (Sigma St. Louis, MO, USA). All other chemicals were purchased from ICN (Irvine, CA, USA).

Cell culture and experimental design. Rat heart cell line H9c2 was obtained from American Type Culture Collection (ATCC Rockville, MD). H9c2 cells were cultured in Dulbecco's modified Eagle's medium at 37 °C in 5% CO_2, and the medium was changed every 2–3 days. The effects of doxorubicin on free radical production, LOX-1 expression, and H9c2 cell apoptosis were examined. H9c2 cells were incubated with H_2O_2 in order to prove the redox sensitivity of LOX-1 expression. The efficacy of pre-treatment with cardioprotective antioxidant agents at preventing H_2O_2 and doxorubicin-induced effects was also tested. The cells were trypsinized and plated in 75-cm^2 plates to measure LOX-1 expression, and in 25-cm^2 flasks for flow cytometry and apoptosis studies. Subconfluent cells (60%) were detached with trypsin and seeded 24 h before treatment. In order to explore the role of LOX-1 expression in H9c2 apoptosis, we first treated cells with doxorubicin to induce LOX-1 expression and then we incubated these cells in various concentrations of ox-LDL to induce LOX-1 pathway activation. In addition, parallel groups of doxorubicin-treated cells were co-treated with ox-LDL and with k-carrageenan (Sigma), a competitive LOX-1 receptor antagonist.

RT-PCR for LOX-1 mRNA expression. Total RNA was extracted by RNeasy column mini kit (Qiagen, Milan, Italy) and was reverse-transcribed into c-DNA using the RevertAid H Minus First Strand c-DNA Synthesis Kit (MBI Fermentas, Lithuania) employing random hexamers. The transcribed cDNA was then used for PCR amplification to estimate the expression of LOX-1 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH).

Two specific primers matching the published sequences were used to identify and amplify LOX-1 (5′-GACTGGATCTGGCATAA AGA-3′ and 5′-CCTTCTTCTGACATATGCTG-3′), and GAPDH (5′-GAGGGGCCATCCACAGTCTT-3′ and 5′-TTCATTGACCTC AACTACAT-3′) [10,11]. The amplified transcripts were analyzed on 2.5% agarose gel using ethidium bromide. The signal intensities of the bands were quantified by densitometric analysis software (Syngene, UK) and compared with the internal standard GAPDH.

Western blot analysis for LOX-1 protein. The H9c2 cells were lysed on ice in a lysis buffer, and the protein concentration of each sample was measured using BCA protein assay reagents. Samples containing 60 μg of total protein were loaded onto a 12% SDS–PAGE and then electrophoretically transferred to a polyvinylidene difluoride membrane [12]. After blocking the membrane with 5% non-fat dried milk in TBST for 1 h at room temperature, it was incubated for 2 h with a polyclonal primary rat LOX-1 antibody (Santa Cruz Biotechnology, CA, USA). After three washings in TBST, the membrane was incubated with a specific peroxidase-coupled secondary antibody for 1 h (Santa Cruz Biotechnology). The membrane was washed again and the signals were visualized by an enhanced chemiluminescence system (Amersham Life Sciences, Arlington Heights, IL). Relative intensities of protein bands were analyzed by densitometry on a gel documentation system with analysis software (Syngene, UK).

Flow-cytometric assay of 2′,7′-dichlorodihydrofluorescein. Determination of intracellular hydroperoxide was based on the oxidation of 2′,7′-dichlorodihydrofluorescein (DCFH) to a fluorescent 2′,7′-dichlorofluorescein (DCF) [13]. DCFH was added at a final concentration of 20 μM and incubated for 30 min at 37 °C. The cells were washed once with PBS and maintained in 1 ml medium. Cellular fluorescence was determined by a flow cytometry apparatus (FACS-SCAN Becton–Dickinson, Franklin Lakes, NJ, USA). Measurements were taken at 510–540 nm after excitation of cells at 488 nm with an argon ion laser.

Preparation of oxidized-LDL. Native LDL (Sigma,1 mg/ml) was oxidized by exposure to $CuSO_4$ (5 μmol/L free Cu^{2+}) in phosphate-buffered saline at 37 °C for 4 h [14]. Control incubations were done in the presence of 200 μM EDTA without $CuSO_4$. Oxidation was terminated by refrigeration. The modified LDL was then dialyzed extensively against 150 mM NaCl containing 1 mM EDTA for 24 h at 4 °C and sterilized by filtration. The extent of the LDL oxidation procedure was determined by evaluating the level of thiobarbituric acid-reactive substances (TBARS) with malondialdehyde as the standard. The TBARS content of oxidized-LDL (Ox-LDL) was 1.10 ± 0.07 vs. 0.24 ± 0.05 nmol/100 μg protein in the native LDL preparation ($p < 0.01$). Protein content was determined by a BCA protein assay kit with the use of bovine serum albumin as the standard. LDL and ox-LDL were kept in 50 mM Tris–HCl, 0.15 M NaCl, and 2 mM EDTA at pH 7.4, and were used within 10 days of preparation.

Flow cytometric detection of apoptosis: FITC-Annexin V/PI double staining. Double staining for FITC-annexin V binding and for cellular DNA using propidium iodide (PI) was performed as follows. After washing twice with PBS, 1×10^6 cells were resuspended in binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$). FITC-Annexin V was added to a final concentration of 1 μg/ml Annexin V. A 0.1 vol. of PI (10 μg/ml in binding buffer) was added, resulting in a final concentration of 1 μg PI/ml cell suspension. The mixture was incubated for 10 min in the dark, at room temperature and then cellular fluorescence was measured by flow cytometry analysis with a FACS-SCAN apparatus.

Statistical analysis. All data represent means ± SD of five separately performed experiments. Statistical comparisons were made with unpaired Student's *t* test or ANOVA with Sheffé's test when appropriate. Statistical significance was set at $p < 0.05$.

Results

Doxorubicin-induced LOX-1 expression in H9c2: effects of carvedilol, atenolol, dexrazoxane, and NAC

LOX-1 is not constitutively expressed in H9c2 cells (Fig. 1). Cultured H9c2 cells incubated with doxorubicin (0.1, 0.5, and 1 μM) for 18 h showed a concentration dependent increase of LOX-1 expression (mRNA and protein). Doxorubicin also caused a time-dependent increase (6-, 12-, and 18-h) of LOX-1 expression (Fig. 1A). Treatment of H9c2 cells with ox-LDL had no effect on LOX-1 mRNA (Fig. 1B). Pre-treatment of cells with carvedilol (10 μM for 1 h) caused a 30% reduction of the doxorubicin-induced LOX-1 expression. The effects of carvedilol were similar to those observed with an antioxidant agent, i.e., NAC (50 μM for 1 h), and with the cardioprotective iron chelating agent, i.e., dexrazoxane (20 μM for 3 h). Pre-treatment with atenolol (10 μM for 1 h), a β-blocker without antioxidant properties, had

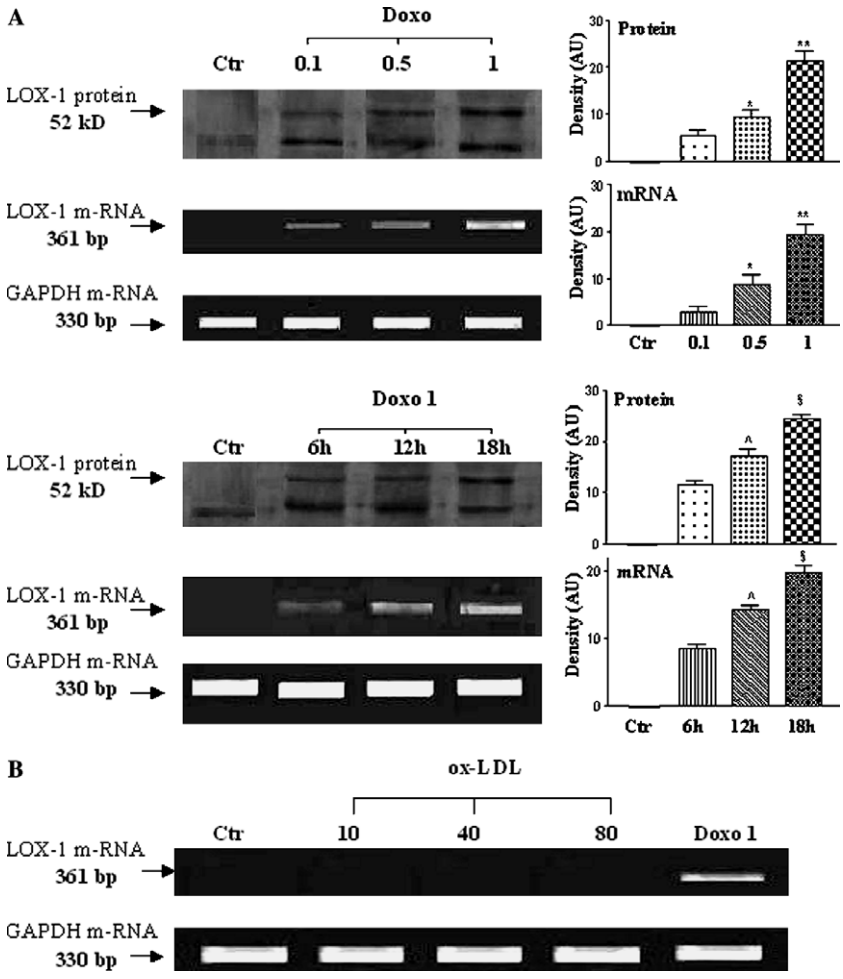

Fig. 1. (A) Effects of doxorubicin on mRNA and protein LOX-1 expression. Incubating H9c2 cells with doxorubicin up-regulates LOX-1 expression in a concentration- and time-dependent manner. Concentrations of doxorubicin are in micromolar. The left panels show the results of a single experiment and the right panels are means ± SD of five separate experiments. Ctr, control. $^{*}p < 0.05$ vs. doxo 0.1, $^{**}p < 0.01$ vs. doxo 0.5, $^{\wedge}p < 0.05$ vs. doxo 6 h, and $^{\S}p < 0.05$ vs. doxo 12 h. (B) Ox-LDL has no effect on mRNA LOX-1 expression. Concentrations of ox-LDL are in μg/ml.

no effect on the LOX-1 expression induced by doxorubicin (Fig. 2).

Hydrogen peroxide up-regulates LOX-1 expression

In order to confirm the role that oxidative stress plays on inducing LOX-1 expression (mRNA and protein), cardiac myocytes were incubated with exogenously administered H_2O_2 for 4 h. LOX-1 mRNA and LOX-1 protein expression levels increased in a dose-dependent fashion (25, 50, and 100 μM). Pre-treatment of cells with Carvedilol, NAC, or dexrazoxane abolished the LOX-1 expression. Pre-treatment with atenolol had no effect on LOX-1 expression (Fig. 3)

Flow cytometric detection of free radical production

Adding 1 μM doxorubicin to H9c2 caused a twofold increase in DCF fluorescence, thus suggesting intracellular production of H_2O_2 and other hydroperoxides. Pre-treatment with 10 μM carvedilol, 20 μM dexrazoxane, and 50 μM NAC nearly eliminated the doxorubicin-induced free radical release, while pre-treatment with atenolol had no significant lowering effect on DCF fluorescence (Fig. 4).

Doxorubicin-induced expression of LOX-1 enhances H9c2 apoptosis

Incubating H9c2 cells for 24 h with various concentrations of ox-LDL (10, 40, and 80 μg/ml) did not induce apoptosis, while exposing cells to doxorubicin did, as demonstrated by the increase from 3% (untreated cells) to 10% in the number of Annexin-positive/propidium-negative fluorescent cells (Fig. 5). In an attempt to assess whether the doxorubicin-induced LOX-1 expression allows ox-LDL to exert pro-apoptotic effects, we first treated H9c2 with

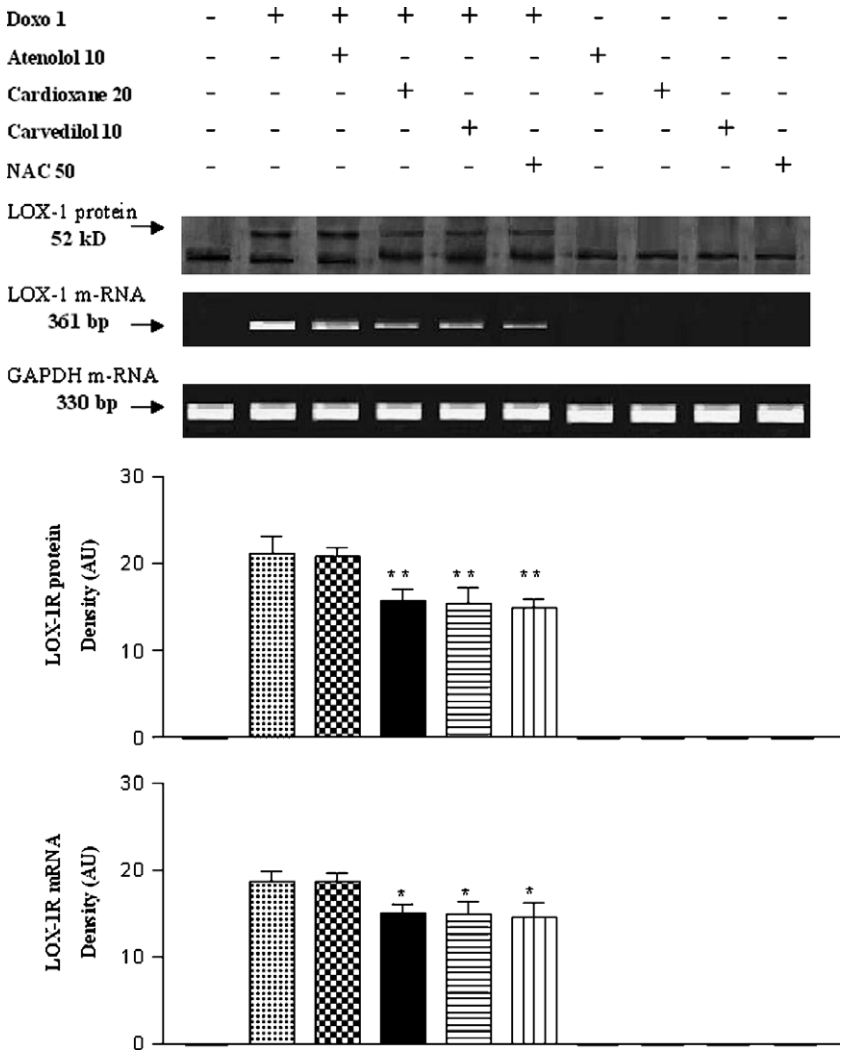

Fig. 2. The doxorubicin-induced LOX-1 expression is decreased by anti-oxidants. Carvedilol, NAC, and dexrazoxane, but not atenolol, significantly lower mRNA and protein LOX-1 levels. The upper panels show the results of a single experiment and the lower panels are means ± SD of five separate experiments. *$p < 0.05$ vs. doxo, **$p < 0.01$ vs. doxo.

doxorubicin 1 μM for 18 h to induce LOX-1 expression, and then we incubated these cells for 24 h in the presence or in the absence of various concentrations of ox-LDL to induce LOX-1 pathway activation. Exposing doxorubicin-treated cells to ox-LDL induced a dose-dependent increase in the fraction of Annexin-positive fluorescent cells (from 10% to 20% in cells exposed to ox-LDL 80 μg/ml). In order to examine whether this increase in cardiac myocyte apoptosis was specifically mediated through the activation of a doxorubicin-induced LOX-1-dependent pathway, parallel groups of doxorubicin pre-treated cells were co-treated with ox-LDL 80 μg/ml and with k-carrageenan 250 μg/ml, a competitive LOX-1 receptor antagonist. Results showed that k-carrageenan significantly reduced the number of Annexin-positive/propidium negative fluorescent cells from 20% to 12% (Fig. 5).

Discussion

This study shows that doxorubicin induces LOX-1 expression in cardiac myocytes and that the expression of this receptor is relevant in doxorubicin-induced apoptosis.

LOX-1 expression is known to be dynamically induced by a variety of stimuli, including its ligand, ox-LDL, as well as by angiotensin, endothelin, inflammatory cytokines, platelets, fluid shear stress, and ischemia–reperfusion [7,15–18]. We observed that LOX-1 expression in H9c2 cells that were exposed to doxorubicin 0.1–1 μM occurs in a concentration- and time-dependent fashion. The effects of higher concentrations of doxorubicin were not tested since we observed that higher concentrations determine necrotic cell death. LOX-1 protein induction peaked at 18 h and was associated with an increase in mRNA, thus

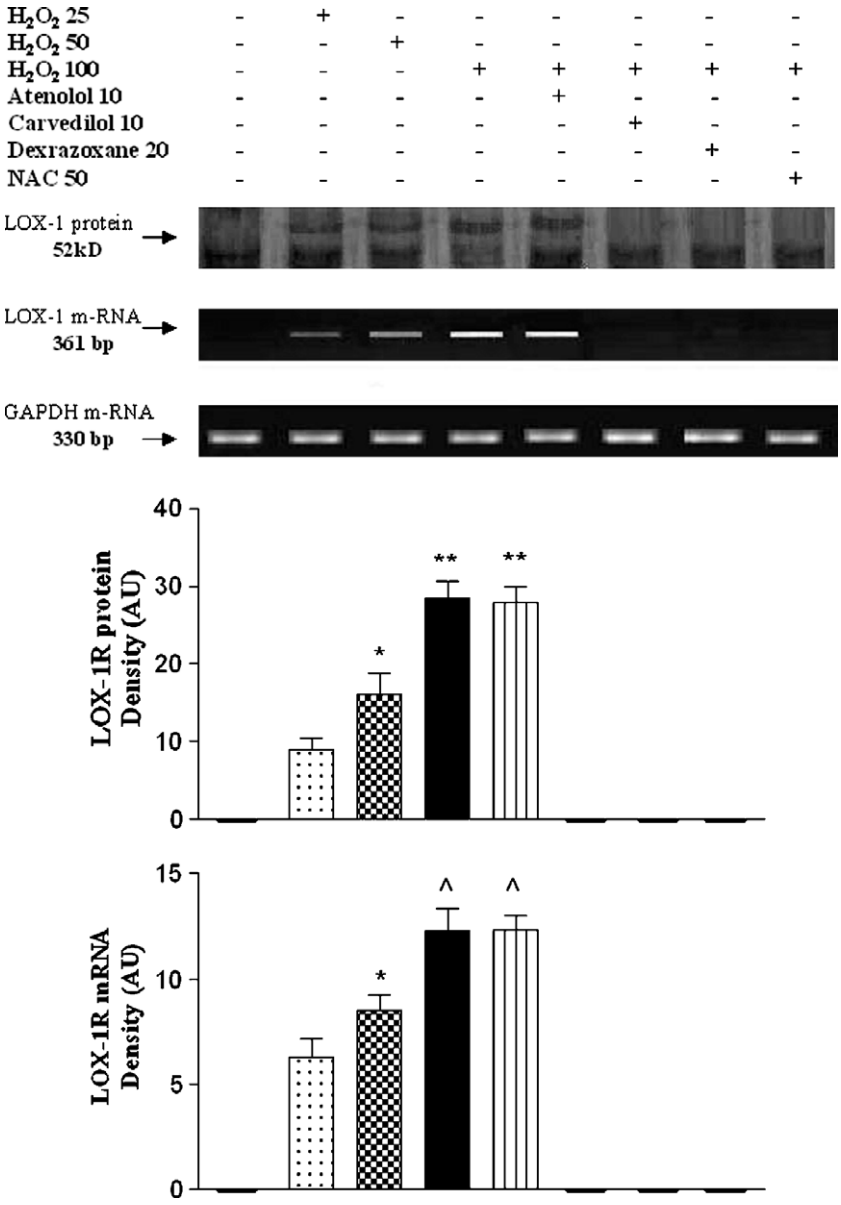

Fig. 3. Dose-dependent induction of LOX-1 expression by H_2O_2. The H_2O_2-induced expression of LOX-1 was abolished by pre-treatment with carvedilol, NAC, or dexrazoxane, but not by pre-treatment with atenolol. Concentrations are in micromolar. Ctr, control. $^*p < 0.05$ vs. H_2O_2 25, $^{**}p < 0.01$ vs. H_2O_2 50, and $\hat{}p < 0.05$ vs. H_2O_2 50.

suggesting transcriptional regulation of LOX-1 by doxorubicin.

Multiple lines of evidence show that there is a link between oxidative stress and LOX-1 expression. First of all, in vivo, LOX-1 expression is up-regulated in hypertension, diabetes, and hyperlipidemia, that is to say in pathological settings that are characterized by enhanced levels of oxidative stress [19,20]. Second, several modified molecules, which are produced by the exposure to oxidative stress, induce LOX-1 expression. This is the case of isoprostanes or ox-LDL itself which induces LOX-1, while native LDL has no effect on LOX-1 expression [15,21,22]. Third, and most importantly, LOX-1 gene expression in endothelial cells is up-regulated by H_2O_2 and by molecules which induce oxidative stress, while antioxidants prevent LOX-1 expression [23,24] . Our data confirm the redox-sensitive regulation of LOX-1 expression in H9c2 cells. In fact, doxorubicin, a drug which increases ROS formation [9], also enhances the transcript levels of LOX-1, and this enhancement is prevented by antioxidants like NAC, dexrazoxane, and carvedilol. Interestingly, atenolol, a β-adrenergic blocker that has no antioxidant activities, has no effect at all in preventing LOX-1 expression.

Growing evidence indicates that up-regulation of LOX-1 is implicated in apoptosis in several cellular types and in various experimental models. Kataoka

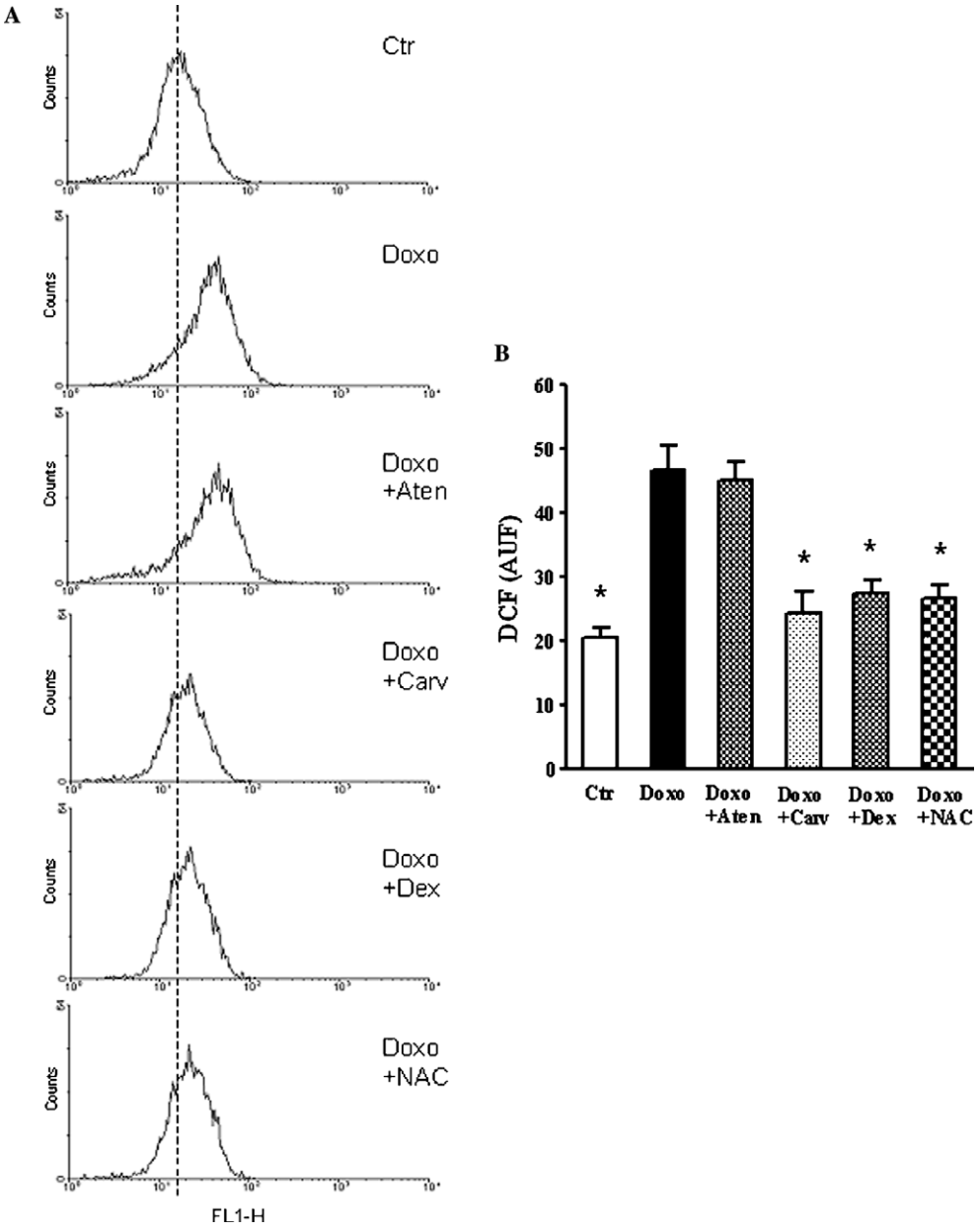

Fig. 4. (A) Flow cytometric histograms of DCF in H9c2 cells. Ctr, control; Doxo, doxorubicin; Aten, atenolol; Carv, carvedilol; Dex, dexrazoxane; and NAC, *N*-acetylcysteine. FL-H relative DCF fluorescence intensity. Data are the results of a single experiment. (B) Column bar graph of cell fluorescence for DCF. Carvedilol, NAC, and dexrazoxane, but not atenolol, significantly blunted the doxorubicin-induced free radical release. Results are means ± SD of five separate experiments. $*p < 0.001$ vs. doxo.

et al. and Chen et al. [2,25] have shown the key role that LOX-1 plays in ox-LDL-induced apoptosis in both vascular smooth muscle cells and in human coronary artery endothelial cells. Using primary neonatal rat cardiomyocytes, Iwai-Kanai et al. [6] demonstrated that a low concentration of ox-LDL, which does not induce apoptosis by itself, results in an increase in the number of TUNEL-positive cells in the presence of norepinephrine or endothelin-induced overexpression of LOX-1. Li et al. [7] proved that LOX-1 up-regulation and activation promotes myocardial cell apoptosis in rat hearts subjected to ischemia for 60 min followed by 60 min of reperfusion.

By using rat heart deriving embryonic cells, we found that ox-LDL does not induce myocardial cell apoptosis by itself, while doxorubicin can induce apoptotic cell death. Interestingly, we also observed that the number of apoptotic cells was significantly higher if treatment with doxorubicin was followed by exposure to ox-LDL, and that administering the LOX-1 antagonist, carrageenan, was protective in cells that had been pretreated with doxorubicin and then exposed to ox-LDL.

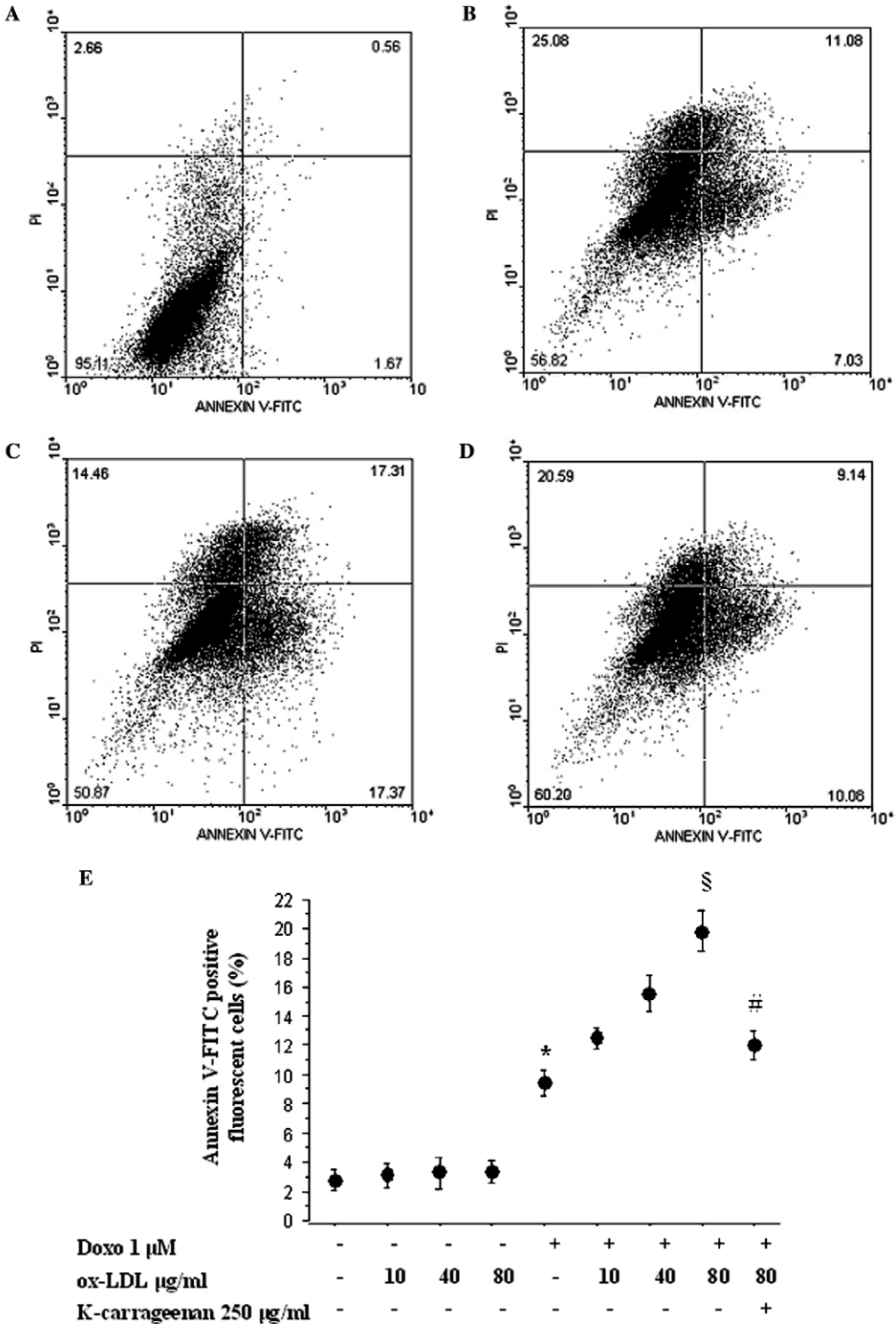

Fig. 5. Flow cytometric analysis of apoptosis in H9c2 cells that were treated with doxorubicin and ox-LDL. The upper panels are diagrams of (A) untreated control cells; (B) cells that were treated with doxorubicin 1 μM; (C) cells that were pre-treated with doxorubicin 1 μM and then incubated with ox-LDL 80 μg/ml; (D) cells that were pre-treated with doxorubicin 1 μM and then co-incubated with ox-LDL 80 μg/ml and k-carrageenan 250 μg/ml. The lower panel (E) shows a cell point chart analysis. Data are expressed as means ± SD based on five separate experiments. $^{*}p < 0.01$ vs. untreated cells; $^{\S}p < 0.01$ vs. doxo 1 μM; $^{\#}p < 0.05$ vs. doxo 1 μM + ox-LDL 80 μg/ml.

Collectively, these data suggest that the doxorubicin-induced LOX-1 up-regulation promotes apoptosis in the presence of ox-LDL.

This study does have some limitations. First, experiments were conducted using H9c2 heart cells which are phenotypically distinct from cardiac myocytes, even

though they share several properties. H9c2 heart cells are recognized as a well-suited model for cardiomyocyte biology, and indeed, they have often been used to study cardiac cell apoptosis secondary to oxidative stress, doxorubicin, energy deprivation, or iron exposure [13,26–28]. Nonetheless, our results should be viewed as hypothesis-generating rather than definitive. Second, ox-LDL, which cannot easily pass through the endothelium, is not the most likely ligand of myocyte LOX-1. However, two studies have reported on the myocardial localization of ox-LDL. Li et al. [7] found a high ox-LDL myocardial content in an experimental model of ischemia–reperfusion. Ekmekcioglu et al. [29] observed that ox-LDLs accumulate in the left and in the ventricular walls of patients with coronary artery disease, or dilated cardiomyopathy, as well as in healthy heart donors, and also found that ventricular accumulation of ox-LDL is a generalized pathophysiological process which does not involve the coronary arteries alone. It is important to bear in mind that LOX-1 does not only bind ox-LDL, but that it also recognizes and is activated by other ligands exposed on the outer surface of apoptotic cells [30]. This would suggest that once doxorubicin has induced LOX-1 expression, the LOX-1 pathway could be activated even in the absence of ox-LDL.

A great deal of effort has been made to identify which agents might mitigate the cardiotoxic effects of doxorubicin. Among the various molecules, dexrazoxane is the only drug that has been approved for clinical use. Recent experimental evidence suggests that pre-treatment with carvedilol might be useful in reducing the incidence and severity of cardiomyopathy associated with doxorubicin administration [13,31]. Carvedilol is known to work as an antioxidant and not as a β-blocker in the prevention of doxorubicin-induced cardiotoxicity, mainly by counteracting the mitochondria-dependent apoptotic pathway [13,31]. The ability of carvedilol, as shown in our study, to down-regulate LOX-1 expression represents a novel mechanism of cardioprotection. Investigations by Li et al., demonstrated that another antioxidant, the hypolipemic drug probucol, is highly effective at preventing cardiac damage in animal models of doxorubicin-induced cardiotoxicity. These effects were interpreted as the ability of probucol to increase intracellular glutathione peroxidase and superoxide dismutase, and thus to maintain the normal antioxidant status of the heart which is disarranged in the course of doxorubicin treatment [32]. In the light of the present data, it could be speculated that probucol may also protect the heart by lowering the plasma levels of LDL, which are known to increase in the course of doxorubicin therapy, and by inhibiting their oxidative modification [33,34].

In summary, we have demonstrated that doxorubicin up-regulates LOX-1 expression in cardiac myocytes, and that LOX-1 pathway activation is involved in the induction of apoptosis. LOX-1 may therefore be a valuable drug TARGET for the prevention of doxorubicin-induced cardiomyopathy.

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