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Cooperative Effects of Q10, Vitamin D_3, and L-Arginine on Cardiac and Endothelial Cells

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Keywords

Q10 · Vitamin D · Nitric oxide · Reactive oxygen species · Endothelial cells · Cardiac cells

Abstract

This work demonstrates the cooperative effect of Q10, vitamin D_3, and L-arginine on both cardiac and endothelial cells. The effects of Q10, L-arginine, and vitamin D_3 alone or combined on cell viability, nitric oxide, and reactive oxygen species productions in endothelial and cardiac cells were studied. Moreover, the involvement of PI3K/Akt and ERK/MAPK pathways leading to eNOS activation as well as the involvement of vitamin D receptor were also investigated. The same agents were tested in an animal model to verify vasodilation, nitric oxide, and reactive oxygen species production. The data obtained in this work demonstrate for the first time the beneficial and cooperative effect of stimulation with Q10, L-arginine, and vitamin D_3. Indeed, in cardiac and endothelial cells, Q10, L-arginine, and vitamin D_3 combined were able to induce a nitric oxide production higher than the that induced by the 3 substances alone. The effects on vasodilation induced by cooperative stimulation have been confirmed in an in vivo model as well. The use of a combination of Q10, L-arginine, and vitamin D to counteract increased free radical production could be a potential method to reduce myocardial injury or the effects of aging on the heart.

Introduction

Vascular endothelial activity is a crucial element with regards to cardiovascular function and its impairment is an early manifestation of atherothrombotic disease [1, 2]. The endothelium acts as a barrier and a regulator of blood vessel activity [2] mainly through nitric oxide (NO), which can stimulate the relaxation of the underlying smooth muscle, leading to vasodilation [2]. NO is produced by vascular endothelial cells in response to different stimuli and acts as a messenger molecule [2], activating guanylate cyclase to enhance cGMP, which in turn causes the relaxation of smooth muscle and vasodilation

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[3]. In addition, NO is able to decrease platelet aggregation and adhesiveness, monocyte adhesiveness, cytokine release [4], and to inhibit LDL oxidation [2, 5]. NO is produced by NO synthases (NOS), which utilize L-arginine as their principal substrate, oxidizing it into L-citrulline and NO [1, 6]. eNOS (the endothelial isoform of NOS) is constitutively expressed in endothelial cells and is responsible for the basal release of NO from the endothelium and for the rapid change into NO flux in response to physical stimuli and molecular agonists [1, 7]. A decrease in endothelial NO production appears in the early phases of atherosclerosis development [4, 8]. Moreover, NO synthesis impairment is involved in a number of cardiovascular diseases, such as peripheral vascular disease, congestive heart failure (HF), and cerebrovascular events [2, 5, 9]. The impaired NO status may cause an overproduction of reactive oxygen species (ROS) in the vasculature, representing a risk factor for cardiovascular disease. To counteract this condition, it is necessary to increase the flux of NO from eNOS activity through supplementation with L-arginine. Numerous studies have shown that acute and chronic supplementation with L-arginine improves NO production by endothelium in individuals with risk factors for atherothrombosis, as well as in individuals with established atherothrombotic disease [1]. For this reason, the extracellular L-arginine concentration is the principal determinant of intracellular L-arginine availability for eNOS [1]. Another important element to maintain ROS production at the basal level is the use of "natural" substances able to modulate metabolic pathways and to treat pathological conditions. A lot of studies suggest that a number of natural compounds, such as coenzyme Q10, vitamin D, and other products included in the nutraceuticals group, are able to target multiple pathways under these pathological conditions [10–12]. For example, with regard to cardiovascular diseases, there is strong evidence that demonstrates the link between oxidative stress and impaired mitochondrial function [13, 14]. CoQ10 is located in the mitochondria, lysosomes, Golgi apparatus, and plasma membranes, and provides an antioxidant action either by direct reaction with free radicals or by the regeneration of tocopherol and ascorbate from their oxidized state [15, 16]. Endogenous synthesis of CoQ10 in the body decreases with age. It has been observed that up to 75% of ischemic heart disease patients exhibit low levels of CoQ10 in the plasma and in the heart in relation to the course of the disease [17, 18]. A long-term therapy with CoQ10 has been shown to decrease HF symptoms, to reduce major adverse cardiovascular events and mortality, and to be safe and well tolerated [19]. CoQ10 has been reported to have many biological effects both in vitro and in vivo [20, 21]. CoQ10 is commonly used for the prevention and treatment of many cardiovascular diseases, such as myocardial infarction, congestive HF, and other drug-induced or disease-induced cardiomyopathies [16, 22, 23]. Moreover, Q10 has shown the ability to prevent oxidative stress, apoptotic cell death, and monocytes cell adhesion in human umbilical vein endothelial cells [24]. On the basis of the results obtained in this research field, it is possible to speculate on the feasibility of preparing a mixture of antioxidants and vitamins able to prevent cardiovascular diseases. Some studies demonstrate that a mix of micronutrients, including vitamin D_3 (VD), significantly reduces markers of lipid peroxidation and at the same time increases the antioxidant potential of the molecules contained. VD, the active form of vitamin D, is not only a regulator of calcium and phosphate homeostasis, but also has numerous extraskeletal effects, such as on the cardiovascular system, central nervous system, endocrine system, and immune system [25, 26]. VD is able to induce its effects through binding to the vitamin D receptor (VDR), which is present in different tissues, including endothelium, vascular smooth muscle, and cardiomyocytes. The importance of VD in the cardiovascular system is proven by the observation that the deletion of VDR from the heart results in hypertrophy [27, 28] and contributes to the acceleration of atherosclerosis [29, 30]. Both experimental and clinical studies support the protective role of vitamin D in the vascular system and in cardiac function in lowering blood pressure, improving endothelial function, inhibiting oxidative stress, and reducing the activity of the renin-angiotensin system [31]. A number of large epidemiologic studies have demonstrated the association between the reduction in 25 OHD plasma concentrations and increased cardiovascular disease risk [30]. Normalization of the VD status could also reduce therapeutic requirements for antihypertensive and cardiac drugs (e.g., diuretics, ACE inhibitors, calcium antagonists) [26]. In humans, the relationship between low serum levels of vitamin D, hypercalcemia, osteoporosis, vascular calcification, and cardiovascular diseases has been extensively studied [32]. For example, in a recent study performed on elderly subjects, an association between low serum vitamin D levels and high arterial blood pressure was found [33]. Additional studies demonstrate the possible autocrine/intracrine mechanisms exerted by vitamin D as a modulator of endothelial functions [32]. In addition, it has been demonstrated that the control on NO production exerted by VD is mediated by VDR and related to intracellular

pathways leading to eNOS activation [32]. Therefore, VDR modulation could represent a new treatment option for all cardiovascular diseases in the future [34].

Thus, in the present study we have examined the effects of the combination of Q10, L-arginine, and VD on cell viability and NO/ROS production in endothelial and cardiac cells in order to assess a potential cooperative effect of these substances on cardiovascular function at the cellular level. Moreover, the involvement of PI3K/Akt and ERK/MAPK pathways leading to eNOS activation has been investigated. To confirm the data, the same agents were also tested in an animal model to assess vasodilation, NO, and ROS production.

Materials and Methods

Cell Culture

Experiments were performed on porcine aortic endothelial (PAE) and rat cardiac (H9c2) cells. PAE cells were purchased from Cell Applications Inc. (San Diego, CA, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma, Milan, Italy) supplemented with 10% fetal bovine serum (FBS; Sigma), 2 mM L-glutamine (Sigma), and 1% penicillin-streptomycin (Sigma) at 37°C with 5% CO_2, as reported in the literature [35]. The cells were plated at 1×10^4 in 96-well plates to analyze cell viability using the MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) test to evaluate NO production and ROS production using Griess assay and the rate of superoxide release, respectively. To study the intracellular pathways, they were plated in a 6-well plate until confluence (about 90%) using Western blot.

H9c2 cells were obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM (Sigma) supplemented with 10% FBS (Sigma), 1% penicillin-streptomycin (Sigma), and 2 mM L-glutamine (Sigma) in an incubator at 5% CO_2 and 37°C, as reported in the literature [36]. The experiments were performed with cells from passages 14–17. The cells were plated at 8×10^3 in 96-well plates to analyze cell viability using the MTT test. NO and ROS production were evaluated using Griess assay and the rate of superoxide release, respectively. Finally, to study the intracellular pathways, cells were plated in a 6-well plate until confluence (about 90%) using Western blot. Before the experiments, both cell lines were maintained in DMEM 0% serum supplemented with L-glutamine and penicillin-streptomycin without red phenol (starvation medium) for 4–18 h.

In vivo Study

Male Wistar rats weighing 350–400 g ($n = 125$) were housed in a room at a constant temperature of 25°C on a 12/12-h light/dark cycle with food and water available ad libitum. All experiments were conducted in accordance with local ethical standards and the protocols were approved by national guidelines (DLGS, January 27, 1992, license 116) and in accordance with the Guide for the Care and Use of Laboratory Animals (National Institute of Health guideline 86-23, 1985 revision). For each animal, anesthesia was performed via sevoflurane (5% for induction and 3% for maintenance) in oxygen [17]. A heat pad maintained the temperature of the animals. Under sterile conditions, rats underwent the surgical procedure of thoracotomy to expose and isolate the aorta in order to perform the injection and to measure blood flow using a flowmeter probe (model 420, Transonic Systems Inc., Ithaca, NY, USA) positioned around the vessel. Aortic blood and tissue were sampled immediately after the end of treatments. Heart rates and respiratory rates were also continuously monitored. The aorta was chosen to study the cardiac function of the drugs based on results from previous studies [37]. Additional experiments were performed to study the effects of QLD after ischemia-reperfusion injury. Ischemia was induced following a classical technique [38], involving clamping isolated aorta downstream of the induction point. The clamp was maintained for 30 min for monitoring of aortic blood flow. After that, the clamp was removed and reperfusion was maintained for 1 h, also with monitoring of aortic blood flow. Finally, during reperfusion, some animals were treated with QLD and monitored for 1 h.

Experimental Protocol

The study was divided into 2 parts, beginning with the in vitro experiments followed by the in vivo experiments. In the in vitro experiments, both cell lines were used to study the cooperative activity of the different biological substances, namely Q10, L-arginine, and VD, to demonstrate the efficacy on vasodilation. Firstly, the influence of the single agents was tested on cell viability in a time-course study (from 30 to 300 s) on H9c2 and PAE cells. The range of concentration of the single agents was reported to exert biological effects: from 2.5 to 50 μM for Q10, from 2.87 to 11.5 mM for L-arginine, and from 10 to 1 μM for VD [20, 39]. Q10 and VD were prepared in ET-OH 100%, then diluted in sterile saline 0.9% solution, and then directly added to the culture medium to obtain the final concentration reported above. L-arginine was directly dissolved in sterile saline 0.9% and then directly added to the culture medium to obtain the final concentration reported above. Secondly, the cooperative activity was demonstrated by adding all agents at the same time (here named QLD) to analyze cell viability, NO production, and ROS production on both cell lines. Finally, the intracellular pathways involved by single agents and QLD were studied by Western blot analysis. In addition, the inhibitors (100 nM wortmannin, 10 μM UO126, and 10 mM L-NAME administered 30 min before the QLD addition) [32] of intracellular pathways were tested on NO production to verify the mechanism of action.

In the in vivo experiments, 130 male Wistar rats (weighing 350–400 g) were used to study blood flow, NO and ROS production, SOD activity, and intracellular pathways. The rats were randomized into 4 groups: $n = 115$ to evaluate the time-course (from 30 to 300 s) of single agents for each concentration, including untreated rats and solvent alone; $n = 10$ to study QLD; $n = 5$ to study ischemia, and $n = 10$ to study QLD after ischemia induction.

Dose selection was based on observations from the in vitro experiments and upon a study on the translation of doses from animals to humans [40]. Thus, Q10 administration resulted in 12.5 mg for in vitro experiments and 50 mg in the in vivo experiments. Similarly, L-arginine administration was 0.5 and 2 g, respectively, and VD was 1.25 and 5 μg, respectively. The agents administered to the rats were prepared in 750 μL of sterile saline 0.9% solution in a similar manner to that reported for the in vitro experiments. Blood samples were obtained from the abdominal aorta with a sterile syringe containing 50 IU/mL of heparin (100 USP units/mL;

Sigma). Finally, the abdominal aorta tract was removed immediately and placed into lysed buffer for Western blot analysis and stored at −80°C prior to extractions.

Plasma Preparation

During sacrifice, blood was withdrawn from the aorta into EDTA-treated tubes, and centrifuged at 1,200 *g* for 10 min at room temperature to obtain the plasma, which was stored at −80°C until use.

Cell Viability

An MTT-based in vitro Toxicology Assay Kit (Sigma) was used to study cell viability [41, 42]. After the treatments, the medium without red phenol and FBS containing the 1% MTT dye was added to 96-well plates containing the cells and incubated for 2 h at 37°C in an incubator. The purple formazan crystals were dissolved in DMSO and cell viability was determined measuring the absorbance through a spectrometer (VICTORX4 Multilabel Plate Reader) at 570 nm with correction at 690 nm. Cell viability was calculated by comparing the results to control cells (100% viable).

Griess Assay

NO production was measured at the end of stimulations through the Griess method (Promega, Milan, Italy) in a 96-well plate on both cell lines maintained in DMEM 0% FBS without red phenol, as previously described [43]. In addition, this assay was also used to measure the level of NO production on plasma samples, as previously described [44, 45], following the manufacturer's instructions.

ROS Production

Superoxide anion production was measured as the superoxide dismutase-inhibitable reduction of cytochrome C, as previously described [41, 42]. In all plasma and cell samples (both stimulated and untreated), cytochrome C was added, and in 1 group superoxide dismutase was also added for 30 min in the incubator (all substances were purchased from Sigma-Aldrich). The absorbance changes in all samples were measured at 550 nm by a spectrometer (VICTORX4 Multilabel Plate Reader). O_2 was expressed as nanomoles per reduced cytochrome C per microgram of protein, using an extinction coefficient of 21,000 mL/cm, after interference absorbance subtraction [41, 42].

SOD Activity

SOD was measured using the Superoxide Dismutase Assay Kit (see online suppl. material; for all online suppl. material, see www.karger.com/doi/10.1159/000484928).

Western Blot

After the stimulations, H9c2 and PAE cells were washed with iced 1× PBS (Sigma) supplemented with 2 mM of sodium orthovanadate (Sigma) and lysed in ice with Complete Tablet Buffer (Roche) supplemented with 2 mmol/L of sodium orthovanadate and 1:1,000 phenylmethanesulfonyl fluoride (PMSF; Sigma-Aldrich). Next, 40 µg from each lysate were resolved on 10% SDS-PAGE gels (Bio-Rad Laboratories, Hercules, CA, USA), transferred to polyvinylidene fluoride membranes (PVDF; GE Healthcare Europe GmbH, Milan, Italy), and incubated overnight at 4°C with specific primary antibodies: anti-phospho-Akt^{Ser473} (1:1,000; Cell Signaling Technologies, Beverly, MA, USA); anti-Akt (1:1,000; Cell Signaling Technologies); anti-phospho-p44/42^{thr202tyr204} (1:1,000; Cell Signaling Technologies); anti-p44/42 (1:1,000; Cell Signaling Technologies); anti-phospho-eNOS^{ser1177} (1:1,000; Cell Signaling Technologies); anti-eNOS (1:1,000; Cell Signaling Technologies); anti-p53 (1:250; Santa-Cruz Biotechnology, Dallas, Texas, USA), and anti-VDR (1:250; Santa-Cruz Biotechnology). Protein expression was normalized and verified through β-actin detection (1:5,000; Sigma).

Tissue Extracts

Aortic tissue samples were homogenized for protein detection (see online suppl. material).

Statistical Analysis

Throughout the study, at least 4 independent experiments were run. Results are expressed as the mean ± SD of 4 technical replicates. One-way ANOVA followed by Bonferroni post hoc test were used for statistical analysis and pairwise differences compared by Mann-Whitney U tests. p values <0.05 were considered statistically significant.

Results

Effects of L-arginine, VD, and CoQ10 on Cell Viability in a Time-Course Study

As presented in Figure 1, Q10, L-arginine, and VD were able to stimulate cell viability on both cell types over the time period. The data showed in particular a difference between low and high concentrations of each substance in a dose-dependent manner, and the higher concentrations of Q10, L-arginine, and VD were shown to have maximum effects compared to the control value ($p < 0.05$). In addition, these concentrations were able to induce an increase in cell viability with a maximum effect after 90 s of stimulation ($p < 0.05$). This is important to demonstrate the physiological activation of cell viability over time. The highest concentrations of Q10, L-arginine, and VD were used for all successive experiments when the time of stimulation was maintained at 90 s.

Effects of Costimulation with L-Arginine, VD, and CoQ10 on NO and ROS Production

Q10, L-arginine, and VD were applied together (referred to in this work as QLD) on H9c2 and PAE cells to demonstrate the ability of these substances to act in a cooperative manner on cell viability, NO, and ROS release. As presented in Figure 2a, the effect of QLD on cell viability in both cell types was time dependent and the maximum effect was observed at 90 s compared to the control (75.5 ± 0.71% in H9c2 and 88.75 ± 0.35% in PAE cells), as previously observed when Q10, L-arginine, and VD were individually tested. Since this study hypothesized the in-

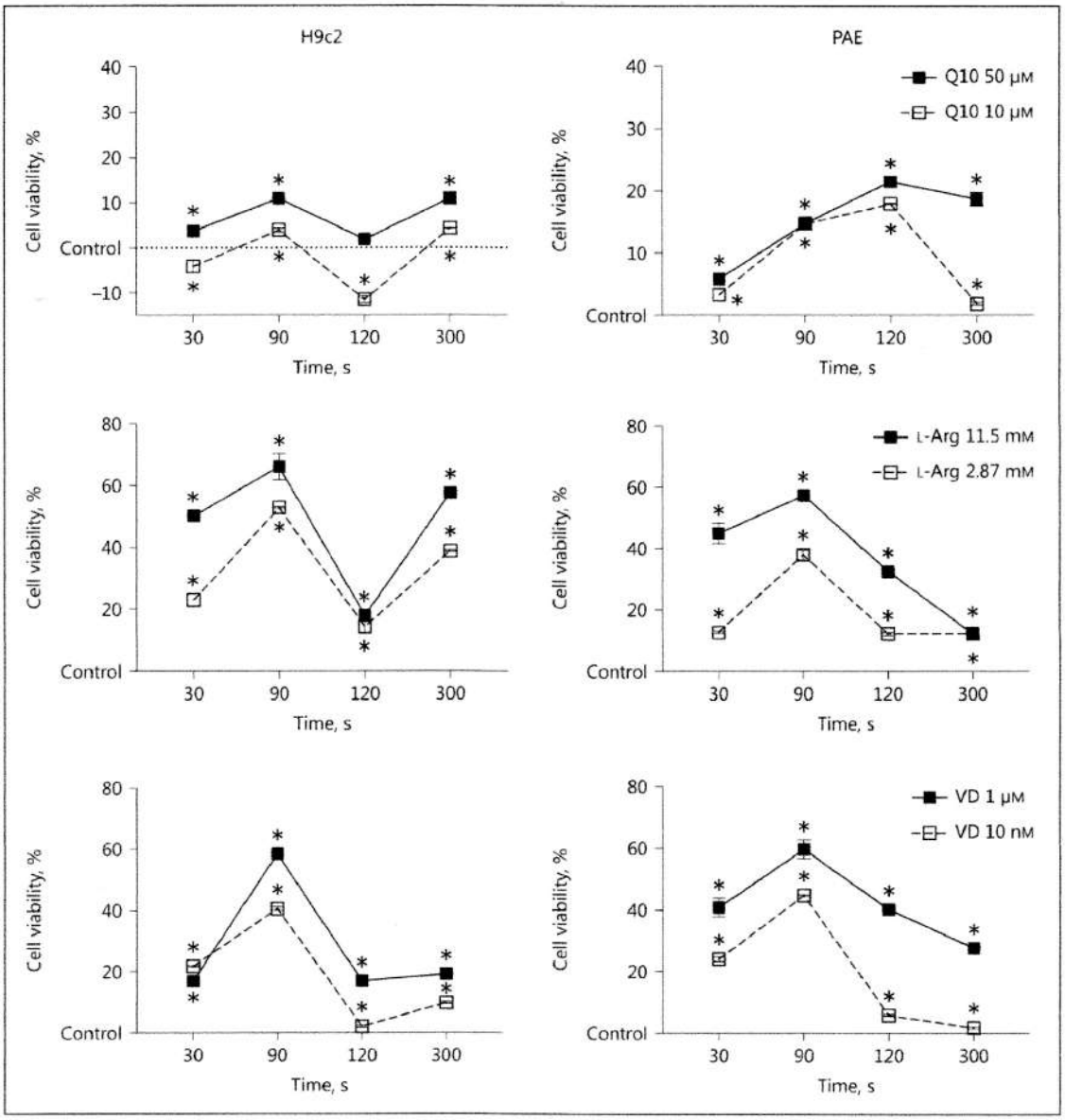

Fig. 1. Time-course and dose-response of Q10, L-arginine, and VD added individually in H9c2 (left) and PAE (right) cells. The concentrations reported correspond to the higher (in black) and the lower (in white) doses used for each substance. Data are the mean ± SD (%) of 5 independent experiments compared to the control (0%). * $p < 0.05$ versus control.

volvement of QLD in vasodilation, NO, and ROS productions were also analyzed. As shown in Figure 2b, NO release was time dependent and the maximal NO release was observed at 90 s on both cell types (4.04 ± 0.08 μM in H9c2 and 2.63 ± 0.18 μM in PAE cells) compared to the control. This production is similar to the physiological release. As shown in Figure 2c, ROS production measured at the same time had an opposite trend, at 90 s the ROS level was less than the control on both cell types (about 42% in H9c2 and 6% in PAE cells). After this time, ROS naturally augmented and NO decreased. In addition, the antioxidant activity of QLD was confirmed by the SOD activity on both cell cultures over time. Figure 2d shows that, at 90 s of stimulation, effects were significant ($p < 0.05$) as compared to the control. All these findings demonstrate for the first time a cooperative effect of QLD on promoting an increase in NO release and a possible role of these substances in vasomotility.

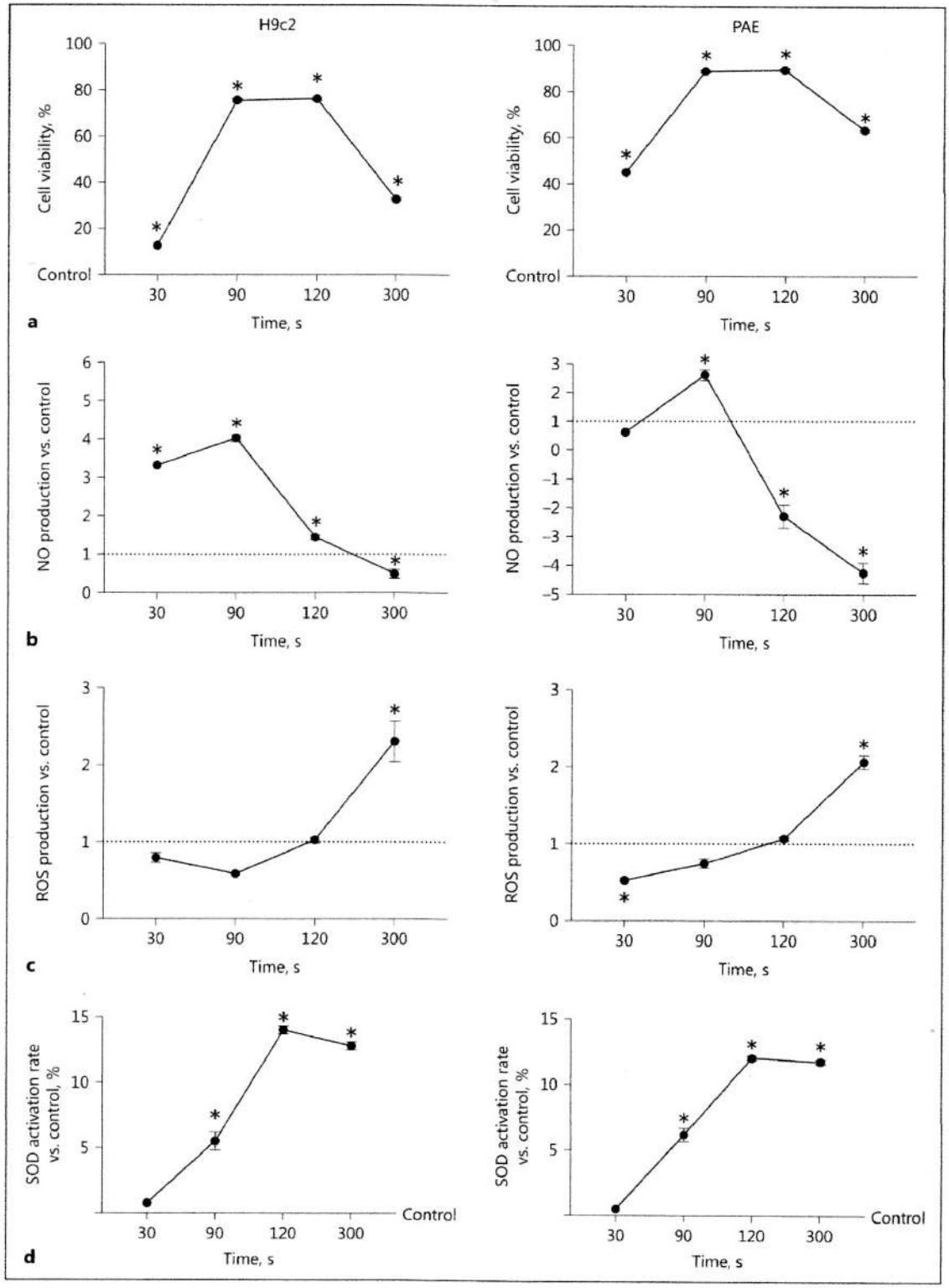

Fig. 2. Cell viability, NO, and ROS production, and SOD activity in H9c2 (left) and PAE (right) cells: time course of Q10, L-arginine, and VD combined, here defined as QLD. **a** Cell viability was shown in both cell types. **b** NO values assessed by the Griess method on cardiac and endothelial cells. **c** ROS production in H9c2 and PAE cells. **d** SOD activity analysis. Data are the mean ± SD (%) compared to the control (0%, **a**, **d**; dotted line, **b**, **c**) of 5 independent experiments. * $p < 0.05$ versus control.

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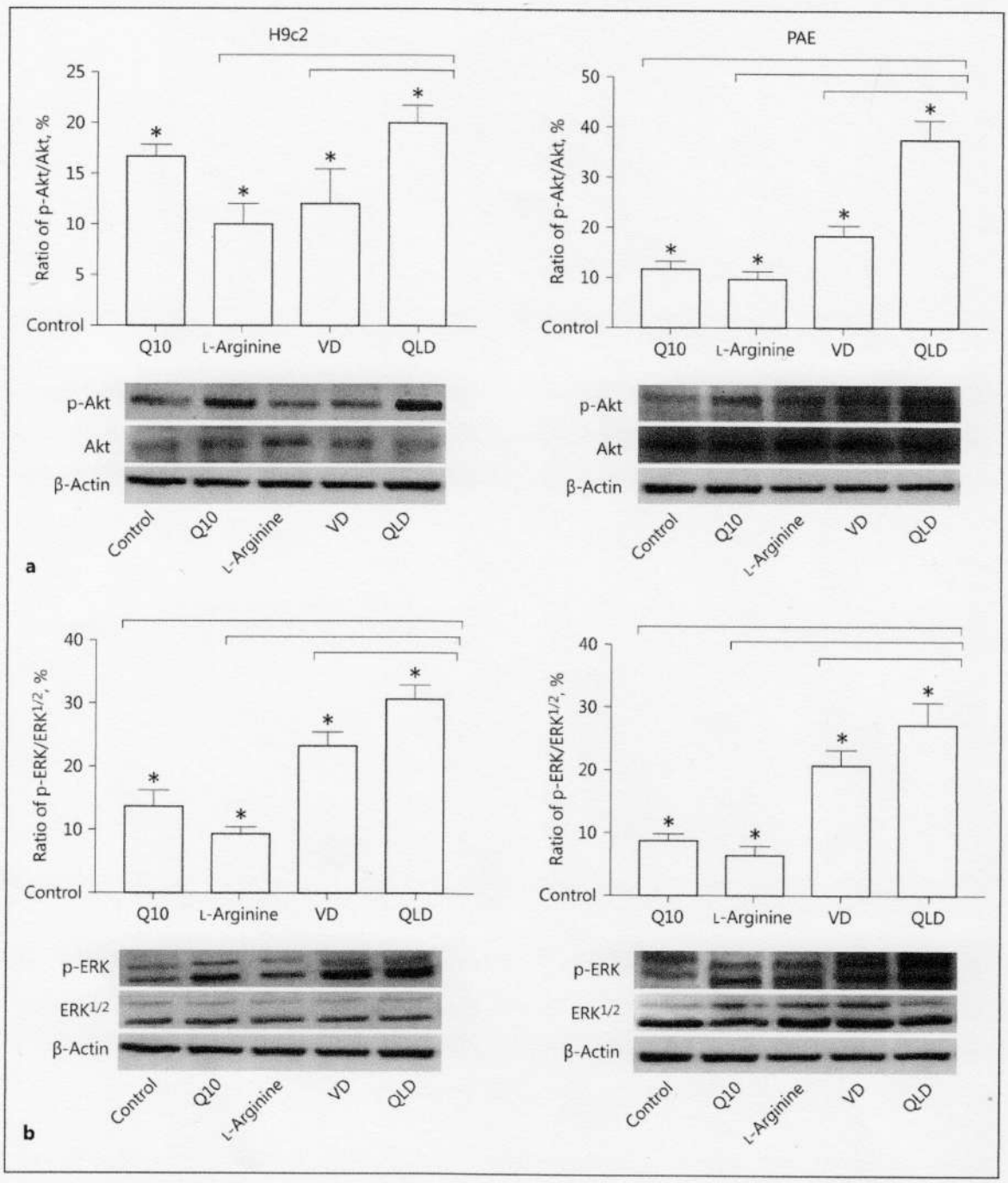

Fig. 3. Western blot and densitometric analysis of Akt (**a**), $ERK^{1/2}$ (**b**), and eNOS (**c**) in H9c2 (left) and PAE (right) cells. An example of the Western blots of each protein is presented. In addition, the densitometric analysis of phosphorylation relative to specific proteins are represented at 90 s of stimulation with Q10, L-arginine, and VD added alone or together (QLD). Data are the mean ± SD (%) of 5 experiments compared to the control (0%). * $p < 0.05$ versus control; bars indicate $p < 0.05$ between QLD and the individual administration.

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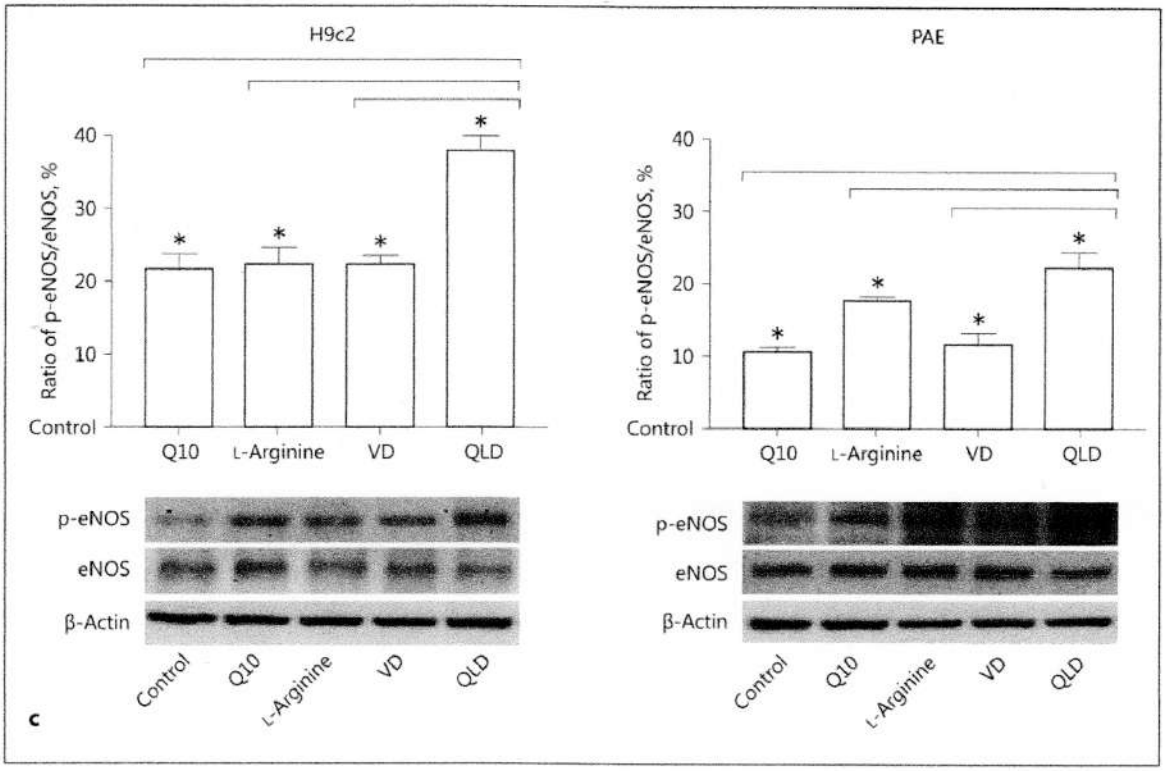

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Intracellular Pathways Involved in NO Release in PAE and H9c2 Cell Lines

The main intracellular pathways involved in vasodilation leading to eNOS activation, such as PI3K/Akt, ERK/MAPK, and other intracellular signaling, such as the VDR receptor and p53, were analyzed in both cell lines by Western blot analysis to explain the mechanisms activated by QLD. As presented in Figure 3, Q10, L-arginine, and VD alone were able to improve ($p < 0.05$) the phosphorylation of Akt, ERK, and eNOS at 90 s in H9c2, and similarly ($p < 0.05$) in PAE cells. Data from the VD experiments confirmed previous findings on its effects on NO production. The Q10 results demonstrated its ability to activate the same mechanism as VD in exerting its beneficial effects ($p < 0.05$). In addition, in the samples treated with QLD, the effect on Akt, $ERK^{1/2}$, and eNOS expressions were amplified ($p < 0.05$) in both cell types compared to the control (about 20, 31, and 38%, respectively, in H9c2 cells; about 38, 27, and 23%, respectively, in PAE cells). These data indicate that QLD was able to activate the intracellular pathways (Akt, $ERK^{1/2}$) leading to eNOS activation with consequent induction of NO release from cardiac and endothelial cells. In addition, the importance of Akt, ERK, and eNOS on NO production and the involvement of these mechanisms was verified using their specific inhibitors (wortmannin, UO16, and LNAME, respectively) in the presence of QLD treatment on both cell types. As shown in online supplementary Figure S1, the pretreatment with the specific inhibitors caused a significant inhibition on NO production ($p < 0.05$) compared to the control and to QLD alone, confirming that QLD acts on Akt, ERK, and eNOS to induce NO release. Since NO production can cause damage when produced at high levels, p53 was analyzed by Western blot in both cell types to exclude any cytotoxic effect. As displayed in Figure 4a, Q10, L-arginine, and VD administered individually did not cause any increase in p53 expression at 90 s of stimulation compared to control values on cardiac and endothelial cells. In particular, VD induced a change of about 7 and 10% in H9c2 and PAE cells, respectively, compared to the control, and Q10 of about 3 and 9% in H9c2 and PAE cells, respectively, compared to the control. In addition, QLD was also able to reduce the expression of p53 compared to the control (about 11 and 12% in H9c2 and PAE cells, respectively), confirming its beneficial effects. Indeed, in response to low levels of oxidative stress, p53 primarily plays an antioxidant role without the involvement of nuclear activation. These findings demonstrate the importance of the cooperative activity of QLD on cardiac and endothelial

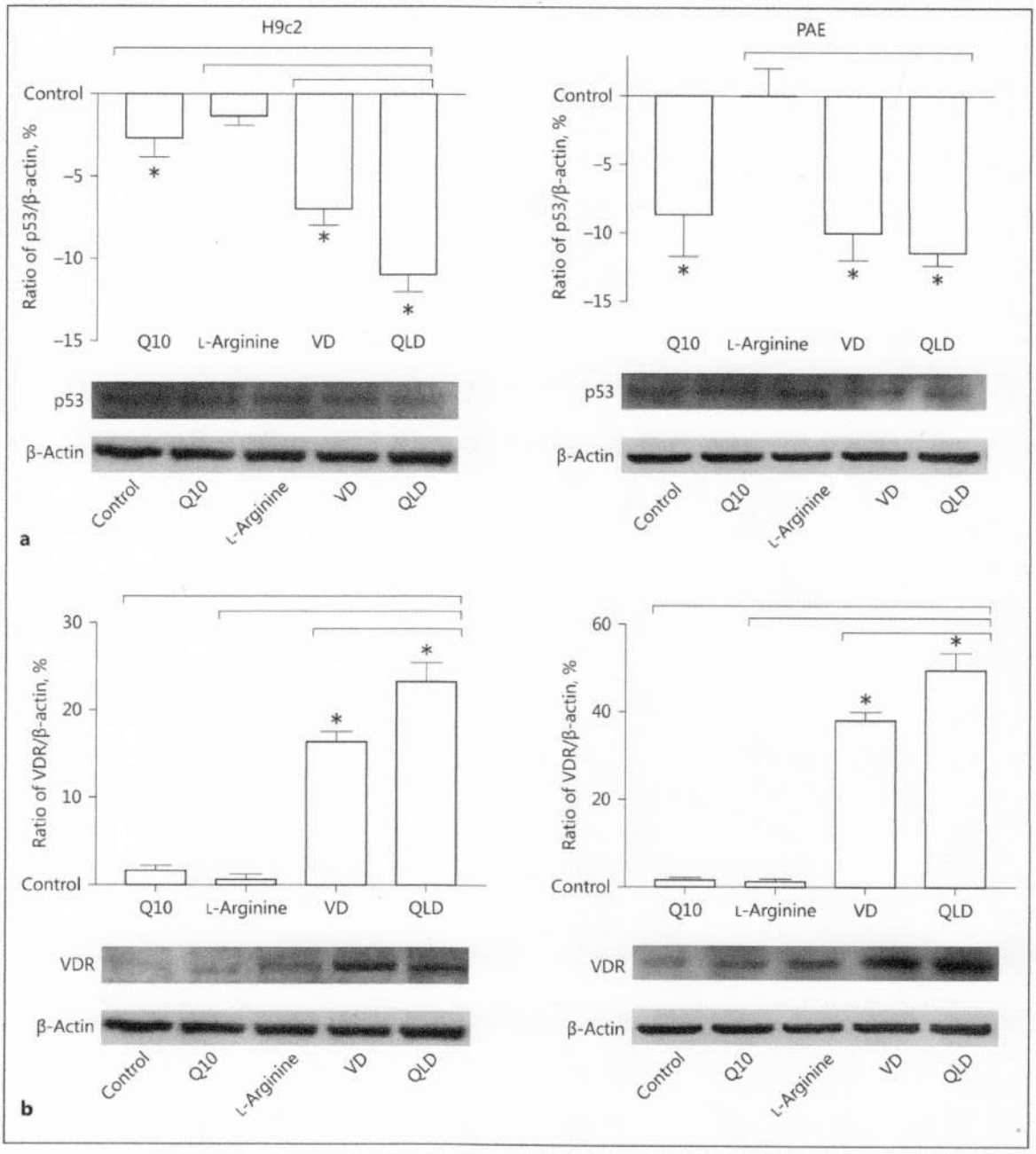

Fig. 4. a, b Densitometric analysis and Western blots of H9c2 (left) and PAE (right) cells of p53 and VDR at 90 s of stimulation with Q10, L-arginine, and VD added alone or together (QLD). The images reported are representative examples. Data are the mean ± SD (%) of 5 experiments compared to the control (0%). * $p < 0.05$ versus control; bars indicate $p < 0.05$ between QLD and the individual administration.

cells. Since QLD includes VD, an analysis of the VDR receptor was considered important to define its role on the positive effect of QLD during vasodilation. Q10 and L-arginine alone were not able to improve the expression of the VDR receptor (Fig. 4b) compared to the control ($p < 0.05$) in both cell types. On the contrary, the stimulation with VD alone was able to induce an enhancement ($p < 0.05$) of VDR expression in cardiac (of about 16%) and endothelial cells (of about 38%) compared to the control, demonstrating the effect of VD in these cell types. In addition, the stimulation with QLD was able to improve this effect of about 23% in cardiac cells and 49%

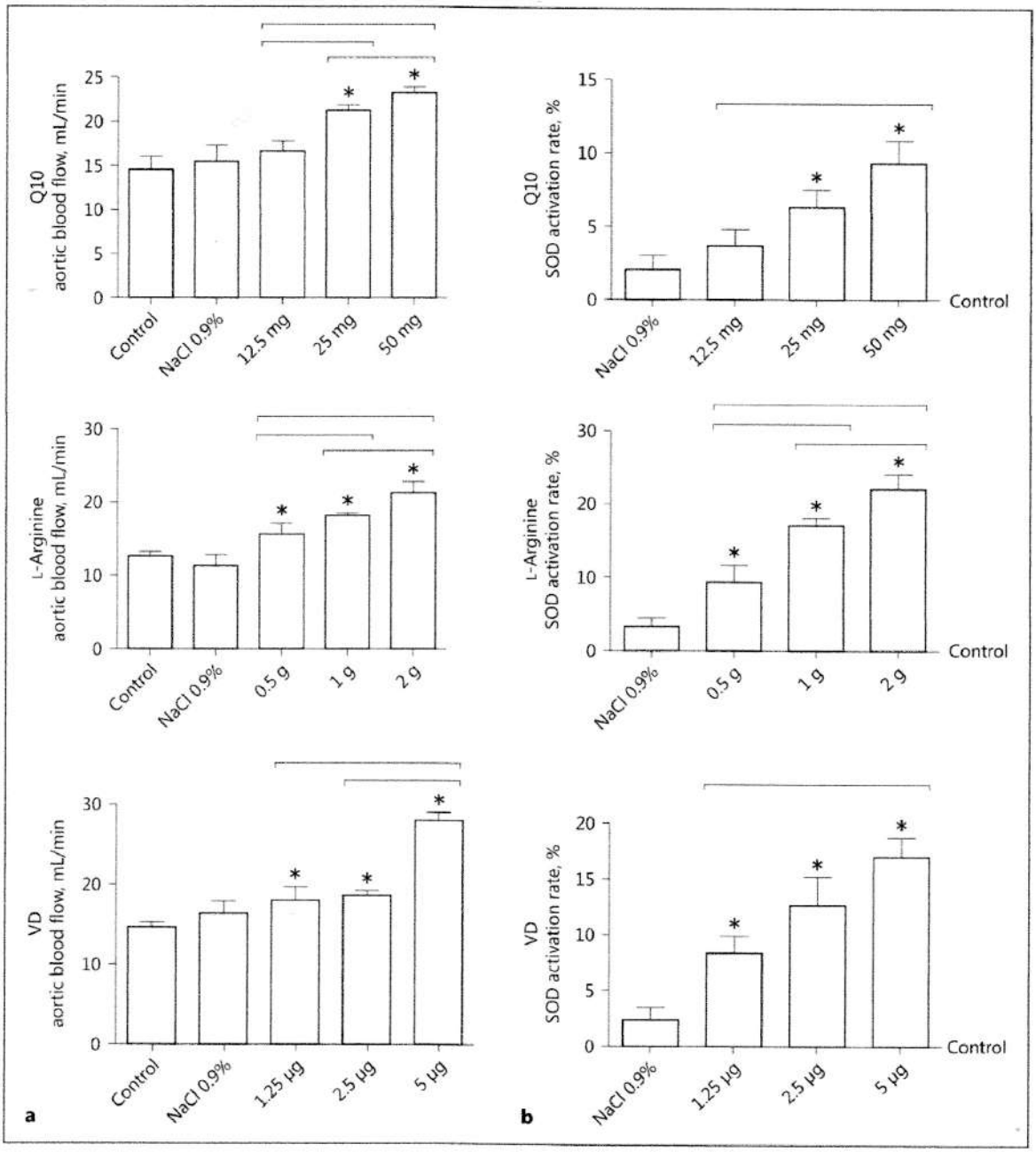

Fig. 5. Aortic blood flow measurements and SOD activity in rats. **a** Vasodilation induced at 120 s by dose-response tests with Q10, L-arginine, and VD individually administered. Data are the mean ± SD (mL/min) of at least 3 rats for each stimulation. The effect of the solvent (NaCl 0.9%) is also shown. **b** SOD activity on plasma samples taken at 120 s by dose-response tests with Q10, L-arginine, and VD individually administered. * $p < 0.05$ versus control; bars indicate $p < 0.05$ between different concentrations of each substance.

in endothelial cells compared to the control, and of 42.5 and 30% compared to VD alone. These findings demonstrate for the first time an improvement of VD effects caused by simultaneous stimulation with Q10 and L-arginine on cardiac and endothelial cells.

In vivo Study of the Effects of Q10, L-Arginine, and VD Combined and Alone

Since the cooperative effect of Q10, L-arginine, and VD acts primarily on vasodilation, these agents were tested either alone or combined in the in vivo experiments.

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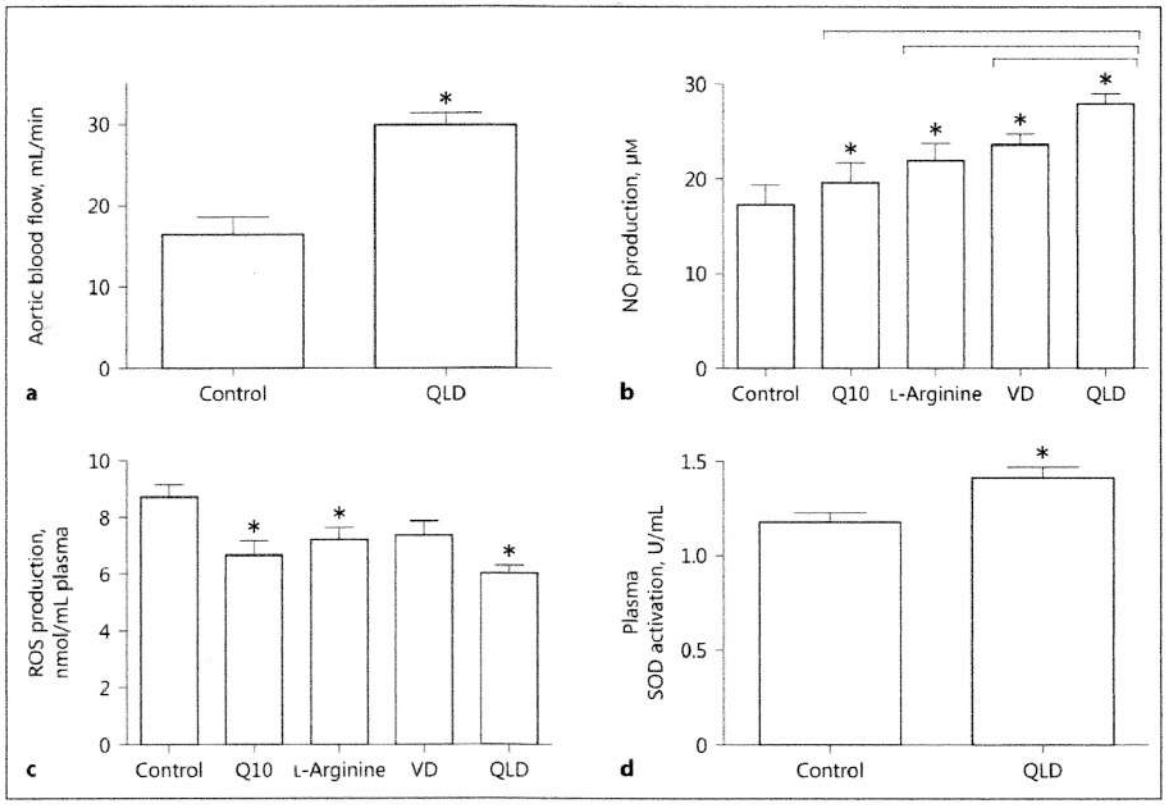

Fig. 6. QLD effects on aortic blood flow (**a**), NO (**b**), ROS production (**c**), and SOD activity (**d**) in plasma rats, reported at 120 s after intravenous administration of QLD. Data are the mean ± SD of at least 4 rats for each stimulation. * $p < 0.05$ versus control; bars (**b**) indicate $p < 0.05$ between QLD and the individual administration.

The single agents were tested in a time-course study and showed a maximal effect on aortic blood flow at 120 s of administration (data not shown). This treatment time was maintained for all successive experiments. An intravenous administration at 120 s showed an increase in aortic blood flow (Fig. 5a) in a concentration-dependent manner ($p < 0.05$) for all substances tested. In particular, the maximum effect was obtained with the maximal concentration used (Q10 about 61%, L-arginine about 200%, VD about 87%) compared to the control and to the minimal doses used (Q10 about 40%, L-arginine about 50%, VD about 56%). These data confirm previous observations on cell culture experiments about the effect of Q10, L-arginine, and VD individually added. In addition, data observed during SOD activity experiments on plasma samples (Fig. 5b) confirmed the data obtained in in vitro experiments about the antioxidant effect. For these findings the maximal concentrations of Q10, L-arginine, and VD was used in all successive experiments. QLD was added to demonstrate the cooperative effect during vasodilation. As presented in Figure 6a, QLD at 120 s can cause an increase of aortic blood flow compared to the control ($p < 0.05$) of about 76% and this effect was bigger than the effect of Q10, L-arginine, and VD alone of about 30, 230, and 7%, respectively. Moreover, another important element involved in vasodilation, NO, was also studied under the same conditions. NO production (Fig. 6b) induced by QLD was significant (62%, $p < 0.05$) compared to the control value, and this production did not cause any damage because at the same time ROS production (Fig. 6c) was reduced ($p < 0.05$) by about 32% compared to the control. Finally, the antioxidant effect (Fig. 6d) of the combination of the 3 substances was confirmed by the analysis of SOD activity on plasma samples ($p < 0.05$): the effect of the combination was greater than the single administration. The importance of the cooperative effect of QLD during vasodilation was also confirmed by Western blot analysis (see online suppl. Fig. S2) in which the effect on Akt, ERK, and eNOS was confirmed and higher than the single administrations ($p < 0.05$). Finally, to verify the effects of QLD, some additional experiments were performed during ischemia-re-

perfusion injury (online suppl. Table S1) in which QLD was seen to restore aortic blood flow ($p < 0.05$ compared to ischemia). All these data demonstrate for the first time a cooperative effect during vasodilation of Q10, L-arginine, and VD under both physiological and pathological conditions.

Discussion

The present study investigated the cooperative effect of Q10, L-arginine, and VD on cardiac and endothelial cells. The major results of this work indicate the importance of QLD on vasodilation through NO production, which was shown to be better than treatments with the individual substances. NO synthesis is involved in a number of cardiovascular diseases, such as peripheral vascular disease, congestive HF, and cerebrovascular events. An impaired NO status may cause an overproduction of ROS in the vasculature, including superoxide anion, hydrogen peroxide, derivatives with hydroxyl radical and hydroxide, lipid peroxides, and derivatives with peroxyl radicals. Q10 suppressed the generation of ROS, which subsequently attenuated the peroxidation and increased the bioavailability of NO [16]. The results of this study demonstrate for the first time that QLD is able to activate eNOS, 1 of 3 isoenzymes transforming L-arginine into L-citrulline and NO, in cardiac and endothelial cells, maintaining the antioxidant properties of the single agents. This finding has been confirmed by SOD assay. This effect is time dependent and is accompanied by a significant increase in the level of phosphorylation of intracellular kinases leading to NO production. Akt kinase activates eNOS by directly phosphorylating the enzyme at Ser-1179 [46]. Akt itself is phosphorylated and activated by PI3 kinase, which in turn is activated by various agonists [47]. Also, MAP kinases, important mediators of signal transduction from the cell surface to the nucleus, have been found to modulate eNOS activation [48]. Indeed, in PAE cells, ERK and Akt pathways have recently been implicated in the effects of various agents on NO production [35, 49]. Data from this study clearly demonstrate that the administration of QLD induced the highest production of NO and acutely increased the phosphorylation of eNOS, Akt, and ERK, which are known to be involved in the intracellular signaling leading to NO production [45, 50, 51]. The relationship between NO and cellular function is complex because NO is cytotoxic at high concentrations and has a protective effect at low concentrations [16]. The importance of Q10, L-arginine, and VD alone on the cardiovascular system is well known. Q10 is an integral component of the mitochondrial respiratory chain for ATP production as well as an antioxidant agent, thus it could assist in improving myocardial function in HF patients [52]. In addition, a meta-analysis on coenzyme Q10 randomized clinical trials showed that it improves the outcomes of HF patients. L-arginine improves the endothelial function modulating the NO bioactivity, increasing intracellular uptake, and acting directly with antioxidant activity [1]. In epidemiological studies, VD deficiency has been consistently associated with an increased risk for cardiovascular disease and hypertension. Disruption of vitamin D signaling in animal models promotes hypertension, cardiac hypertrophy, and atherosclerosis. This evidence has led to prospective randomized trials on VD supplementation in individuals at risk of cardiovascular disease [53]. In the animal model as well as in cell lines, we have investigated the effects of antioxidant therapy with Q10, L-arginine, and VD to improve endothelial and cardiac function by means of a VDR-mediated reduction in ROS production. This finding is supported by the fact that VDR is expressed throughout the myocardium on various cell types, including cardiomyocytes and endothelial cells [45, 54]. QLD was able to maintain the ability of VD to induce its beneficial effects on the cardiovascular system, whereas VD supports the effects of Q10 during vasodilation as a consequence of the L-arginine effect. In addition, the positive effects exerted by QLD were demonstrated thanks to a reduction of p53 activation and ROS production on both cell types. Indeed, the level of ROS observed at 300 s was not enough to cause tissue damage and always remained within the physiological range. In addition, the intracellular pathways leading to NO production observed in in vitro studies were confirmed by Western blot analysis on aortic samples. Since the translational approach is very important to confirm the mechanisms observed in vitro, some experiments were performed in an in vivo model. In rats, the beneficial effects on vasodilation of Q10, L-arginine, and VD combined were confirmed, also when compared to these substances alone. In addition, the effects of QLD on vasodilation were demonstrated through the increase of NO production.

All these data confirm the importance of Q10, L-arginine, and VD during vasodilation, and demonstrate for the first time a possible cooperative use in amplifying the positive effect of each of them. Using QLD to counteract increased free radical production is a potential

method to reduce myocardial injury in patients and to reduce aging effects on the heart. Indeed, the analysis of blood flow during ischemia-reperfusion injury in the in vivo model confirmed the ability of QLD to restore the physiological level of aortic blood flow. This is relevant in the light of the possible use of QLD supplementation as a potential tool to treat or prevent cardiovascular diseases.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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