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Original Contribution

Curcumin protects cardiac cells against ischemia-reperfusion injury: effects on oxidative stress, NF-κB, and JNK pathways

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ABSTRACT

In this study we explored the effects of curcumin in cardiac cells subjected to a protocol simulating ischemia-reperfusion (IR). Curcumin (10 μM) was administered before ischemia (pretreatment) or at the moment of reperfusion (posttreatment) and its effects were compared to those produced by a reference antioxidant (Trolox) with an equal antioxidant capacity. IR cardiac cells showed clear signs of oxidative stress, impaired mitochondrial activity, and a marked development of both necrotic and apoptotic processes; at the same time, increases in NF-κB nuclear translocation and JNK phosphorylation were observed. Curcumin pretreatment was revealed to be the most effective in attenuating all these modifications and, in particular, in reducing the death of IR cells. This confirms that the protective effect of curcumin is not related simply to its antioxidant properties but involves other mechanisms, notably interactions in the NF-κB and JNK pathways. These findings suggest that curcumin administration, in particular before the hypoxic challenge, represents a promising approach to protecting cardiac cells against IR injury. In this scenario our results point out the importance of the chronology for the outcome of the treatment and provide a differential valuation of the degree of protection that curcumin can exert by its antioxidant activity or by other mechanisms.
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Ischemia-reperfusion (IR) injury, a condition that plays an important role in several circumstances of clinical interest, is multifactorial, but it is generally accepted that an excessive production of reactive oxygen species (ROS) plays a prominent role in its development [1]. In heart muscle IR has been found to induce myocyte necrosis and apoptosis that seem to be the prevalent mode of death during, respectively, the ischemic period and the reperfusion [2–4]. The intracellular signaling pathways leading to cardiomyocyte death in IR are poorly understood; however, there is evidence that the nuclear factor κB (NF-κB), a pivotal transcription factor [5], is rapidly activated by ischemia in several tissues, including myocardium [6]. In the inactive state NF-κB is present in the cytoplasm as a heterotrimeric complex consisting of two subunits and an additional inhibitory subunit: IκBα. During the activation process, the inhibitory subunit is phosphorylated in Ser 32 and Ser 36 residues by IKK (IκBα kinase) and is subsequently degraded. Once released, subunits of activated NF-κB translocate to the nucleus and activate the expression of selected target genes, among them those involved in cell death pathways [7]. Another pathway that might have a part in cardiomyocyte death during IR is that related to the c-Jun N-terminal kinase (JNK), which represents one of the "stress-responsive" members of the mitogen-activated protein kinase (MAPK) family [8]. A variety of stress stimuli, including oxidative stress, can induce JNK phosphorylation and the consequent activation of this pathway that, by wide consent, is associated with apoptosis [9].

Obviously, antioxidant treatments have a very important role in cell protection against IR injury and many substances have been proposed for this purpose. In the past years growing interest has been addressed to curcumin, a major active component of the food flavor turmeric, which is extracted from the powdered dry rhizome of *Curcuma longa Linn* (Zingiberacee), a perennial herb widely cultivated in tropical regions of Asia. Curcumin, used as a spice to give the specific flavor and yellow color to curry, has been employed for centuries in indigenous medicine for the treatment of a variety of diseases [10]. Several studies in recent years have shown that curcumin is an inhibitor of tumor initiation in vivo [11] and possesses antiproliferative activity against tumor cells in vitro [12]. In addition to these anticarcinogenic properties, curcumin seems to be, even at relatively low concentrations, an effective anti-inflammatory agent [13,14]. Although the exact mechanism by which curcumin promotes these effects remains to be elucidated, the antioxidant properties of this substance seem to be an essential component underlying its pleiotropic biological activities. Curcumin, in fact, has been reported to inhibit lipid peroxidation and to effectively scavenge superoxide anion and hydroxyl radicals. In addition to this antioxidant ability,

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curcumin has been found to inhibit NF-κB activation, and studies in animal models have shown that this effect was associated with a protection against various stressing conditions [15].

These observations caused us to perform the present study, aimed at assessing the effectiveness of curcumin in protecting cardiac cells from IR injury and to explore the molecular mechanism(s) of its action. In particular, we intended to establish if the effects of curcumin were ascribable solely to its antioxidant properties or also to other mechanisms, notably to possible interactions in the NF-κB- and/or JNK-related pathways. To this end the effects of curcumin on cultured cardiomyoblasts subjected to simulated IR were compared to those produced, at a parity of antioxidant capacity, by Trolox, the well-known water-soluble analogue of vitamin E.

Materials and methods

Cell cultures

Rat heart myoblasts (line H9c2 (2-1) n. 88092904, purchased from the European Collection of Cell Cultures) were plated at a density of 5×10^5/100-mm plate and cultured at 37°C in a humidified atmosphere with 5% CO_2 in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, 1% streptomycin, and 1% L-glutamine. Cells were passaged regularly and subcultured to ~90% confluence before experimental procedures. Curcumin, Trolox, Bay 11-7082, and SP600125 were purchased from Sigma. All other reagents were of the highest purity available.

Simulated ischemia-reperfusion protocol and cell treatment

Hypoxic challenge was achieved by using a modular incubator chamber (Billups-Rothenberg, Inc., CA, USA) gassed with 95% N_2, 5% CO_2. A flow meter was used to measure the quantity of gas mixture introduced into the chamber (25 L/min) according to Namiki et al. [16]. To simulate IR, after incubation under hypoxic conditions and in serum and glucose-free medium for 24 h, the cells were reoxygenated by immediate replacement of fresh complete culture medium and were returned to a normoxic environment at 37°C for 60 min. Cell treatment was induced 1 h before the hypoxic challenge or at the moment of reoxygenation by adding curcumin or Trolox at concentrations of respectively 10 and 25 μM, which we found to display the same antioxidant capacity (see below). To check for possible toxic effects of these compounds the normoxic control H9c2 cells were also subjected to the same treatments. In another series of experiments aimed at exploring the contribution of NF-κB and JNK pathways in this experimental model, cells were treated for 1 h before hypoxia with 10 μM Bay 11-7082 (specific inhibitor of NF-κB) or 10 μM SP600125 (specific inhibitor of JNK).

Preparation of cell homogenates

H9c2 cells (1×10^6) were washed twice with phosphate-buffered saline (PBS), trypsinized, collected by centrifugation, and then resuspended in 100 μl of lysis buffer containing 1% Triton X-100, 20 mM Tris-HCl, pH 8, 137 mM NaCl, 10% (v/v) glycerol, 2 mM EDTA, and 6 M urea supplemented with 0.2 mM PMSF as well as 10 μg/ml leupeptin and aprotinin. To obtain cellular homogenates, samples, after three freeze-thaw cycles, were sonicated twice in ice for 5 s and centrifuged at 14,000g for 10 min at 4°C. The supernatant was then collected. Protein content was measured by the Bradford method [17].

Determination of intracellular ROS

Cells were cultured on a 96-well plate and after hypoxia dye loading was achieved by adding to the cell culture medium the ROS-sensitive fluorescent probe CM-H_2DCFDA dissolved in 0.1% DMSO (2.5 μM) and pluronic acid F-127 (0.01% w/v) for 10 min at 37°C. The CM-H_2DCFDA esterified derivative is loaded more effectively within the cytoplasm of the cells because it is more cell permeant before the ester groups are hydrolyzed by the cellular esterases. Only a negligible leakage of the probe occurred, because chloromethyl-DCF is negatively charged at physiological intracellular pH. Fluorescence intensity was analyzed by a fluorescence reader (Fluoroscan Ascent FL, Labsystems, Finland) using a 485-nm excitation and 538-nm emission filter.

Total antioxidant capacity (TAC)

Intracellular TAC, accounting for total hydrophilic ROS scavengers, was measured in cell lysates by a chemiluminescence assay using the photoprotein Pholasin (Abel Antioxidant Test Kit, Knight Scientific Ltd, UK). After being washed with PBS, cells were resuspended in 20 mM Tris-HCl buffer, pH 8, containing 1.0% Triton, 137 mM NaCl, 10% glycerol, 6.0 M urea, 0.1 mM PMSF, 10 μg/ml leupeptin, and 10 μg/ml aprotinin before storage at −80°C until use. Cell plasma membranes were broken by three freeze-thaw cycles, 5.0 s sonication in ice, and centrifugation at 14,000g for 10 min at 4.0°C. Protein content was measured by the Bradford method [17]. The results were calculated using an L-ascorbic acid-based standard curve. The above chemiluminescence assay was used, in an in vitro assay, to assess the antioxidant capacity of curcumin and Trolox.

Lipid peroxidation

To assess the rate of lipid peroxidation, isoprostane levels were measured in cell lysates using the 8-isoprostane EIA kit (Cayman Chemical Co., Ann Arbor, MI, USA) following the manufacturer's instructions. Lipid peroxidation was also investigated by confocal scanning microscopy analysis, using the fluorescent probe BODIPY, which is intrinsically lipophilic, thus mimicking the properties of natural lipids [18]. In particular, BODIPY 581/591 C_{11} acts as a fluorescent lipid peroxidation reporter that shifts its fluorescence from red to green when challenged with oxidizing agents. Briefly, cells were cultured on glass coverslips and dye loading was achieved by adding to the cell culture medium the fluorescent probe BODIPY dissolved in 0.1% DMSO (5 μM final concentration) for 30 min at 37°C. The cells were fixed in 2.0% buffered paraformaldehyde for 10 min at room temperature and the BODIPY fluorescence was analyzed using the confocal Leica TCS SP5 scanning microscope (Mannheim, Germany) equipped with an argon laser source for fluorescence measurements, using a 581-nm excitation wavelength. The observations were performed by collecting the emitted fluorescence with a Leica Plan Apo 63×oil immersion objective. A series of optical sections (1024×1024 pixels) 1.0 μm in thickness was taken through the cell depth at intervals of 0.5 μm. A number of optical sections ranging from 10 to 20 for each examined sample were then projected as a single composite image by superimposition.

Mitochondrial activity assay

Mitochondrial activity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in a 96-well plate [19]. After the cells were washed with PBS, 100 μl of 0.5 mg/ml MTT solution in PBS was added to the cell cultures and the samples were incubated for 4.0 h at 37°C. Finally, 100 μl of cell lysis buffer (20% SDS, 50% *N*,*N*-dimethylformamide, pH 4.7) was added to each well and the samples were incubated for at least 3 h at 37°C in a humidified incubator before determination of absorbance of blue formazan at 590 nm with an ELISA plate reader. Cell viability was expressed as percentage of MTT reduction.

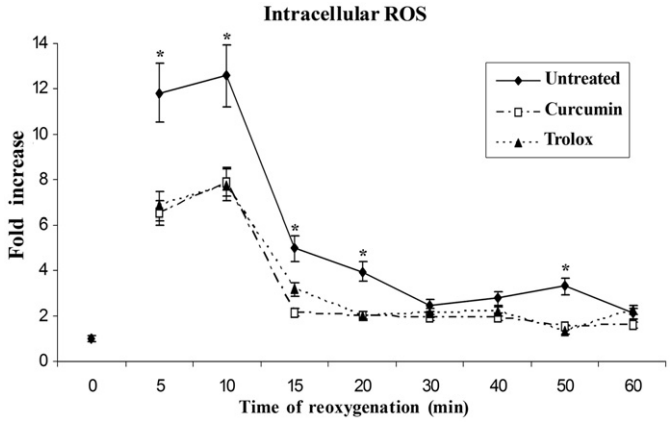

Fig. 1. Effects of curcumin and Trolox pretreatment on intracellular ROS production in IR H9c2 cells at various times of reoxygenation. ROS concentration was measured by CM-H_2DCFDA fluorescence and expressed as fold increase with respect to the control value indicating ROS levels in normoxic control cells. For details, see Materials and methods. All values (means ± SD) are representative of five independent experiments, each carried out in triplicate.

Lactate dehydrogenase (LDH) measurement

LDH activity was assessed spectrophotometrically in the culture medium and in adherent cells (to obtain total LDH content) using the LDH assay kit (Roche Diagnostics, Mannheim, Germany). LDH release was calculated as a percentage of total LDH content.

Assessment of cell death

Mitochondrial permeability was measured by calcein fluorescence according to the method of Petronilli et al. [20] with minor modifications. In brief, calcein/AM freely enters the cell and becomes fluorescent upon deesterification. Coloading of cells with cobalt chloride quenches the fluorescence in the cell except in the mitochondria, because cobalt cannot cross mitochondrial membranes (living cells). During induction of the MPTP (Mitochondrial Permeability Transition Pore) opening cobalt can enter the mitochondria, where it is able to quench calcein fluorescence (apoptotic cells). Thus, decreased mitochondrial calcein fluorescence can be taken as a measure of the extent of MPTP induction. The detection of necrotic cells was performed by staining with propidium iodide. Cells were cultured on glass coverslips and dye loading was achieved by adding to the cell culture medium the fluorescent probes calcein /AM (3 μM), propidium iodide solution (5 μM), and cobalt chloride (1 mM) for 20 min at 37°C.

After cobalt quenching, cells were washed with PBS and fixed in 2.0% buffered paraformaldehyde for 10 min at room temperature and the fluorescence was analyzed using the confocal Leica TCS SP5 scanning microscope equipped with an argon laser source for fluorescence measurements, using 488-nm and 543-nm excitation

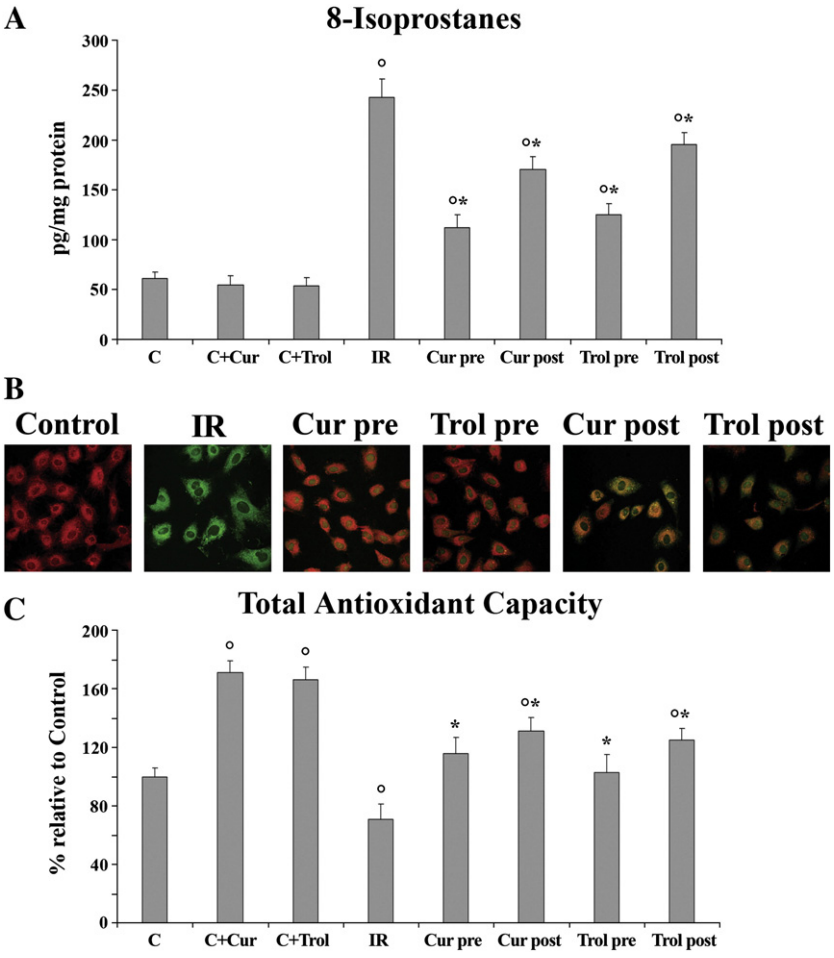

Fig. 2. (A) Rate of lipid peroxidation as measured as cytosolic level of 8-isoprostanes according to Materials and methods. The reported values (means ± SD) are representative of five independent experiments carried out in triplicate. °Significant difference ($p \leq 0.05$) vs control (C) cells. *Significant difference ($p \leq 0.05$) vs IR cells. (B) Representative confocal microscopy images of lipid peroxidation in IR H9c2 cells loaded with the fluorescent probe BODIPY according to Materials and methods. (C) Cellular TAC, expressed in ascorbate equivalent units, was measured by a chemiluminescence assay in the cytosolic fractions of the lysates of IR H9c2 cells exposed or not to 10 μM curcumin or 25 μM Trolox for 1 h before the hypoxic challenge.

wavelengths. The observations were performed by collecting the emitted fluorescence with a Leica Plan Apo 63×oil immersion objective. A series of optical sections (1024×1024 pixels) 1.0 μm in thickness was taken through the cell depth at intervals of 0.5 μm. A number of optical sections ranging from 10 to 20 for each examined sample were then projected as a single composite image by superimposition.

Caspase-3, caspase-8, and caspase-9 activity

Cells were cultured on glass coverslips and the culture medium was removed and replaced with FAM-FLICA caspase-3 and -7 solution (Caspase 3 and 7 FLICA kit FAM-DEVD-FMK; Immunochemistry Technologies, Bloomington, MN, USA), FAM-FLICA caspase-8 solution (Caspase 8 FLICA kit FAM-LETD-FMK), and FAM-FLICA caspase-9 solution (Caspase 9 FLICA kit FAM-LEHD-FMK), for 1 h, following the manufacturer's instructions. Cells were washed three times with a wash buffer provided by the kit and fixed in 2.0% buffered paraformaldehyde for 10 min at room temperature. Fluorescence was detected using the confocal Leica TCS SP5 scanning microscope equipped with an argon laser source for fluorescence measurements, using 488-nm excitation wavelength. The observations were performed by collecting the emitted fluorescence with a Leica Plan Apo 63×oil immersion objective. A series of optical sections (1024×1024 pixels) 1.0 μm in thickness was taken through the cell depth at intervals of 0.5 μm. A number of optical sections ranging from 10 to 20 for each examined sample were then projected as a single composite image by superimposition.

NF-κB nuclear translocation assessment by Western blot

To assess the protein levels of NF-κB, equal amounts of nuclear fraction (60 μg) were diluted in Laemmli's sample buffer with 6 M urea and boiled at 65°C for 5 min. Proteins were separated on 10% SDS-PAGE and were transferred to PVDF Hybond membrane (Millipore Corp., Billerica, MA, USA). The membrane was then incubated overnight at 4°C with a highly specific (mouse) anti-NF-κB (p50 subunit; sc-8414) monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). After being washed, the membrane was incubated with peroxidase-conjugated secondary antibodies for 1 h. Then immunolabeled bands were detected using a SuperSignal West Dura (Pierce, Rockford, IL, USA) and quantified by densitometry analysis using a Chemi Doc system and Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). Results were expressed as ratios between the densitometry of the protein of interest and that of the loading control (histone H1; highly specific anti-histone H1 antibodies were purchased from Abcam plc, Cambridge, UK).

JNK levels

H9c2 cardiac cells were grown to 80% confluence in 96-well tissue culture plates and then subjected to the above-described procedure in the presence or absence of curcumin or Trolox. After cell fixation by 4% formaldehyde in PBS, total JNK and phosphorylated JNK were quantified using a commercially available assay (JNK ELISA kit; FACE, Active Motif Europe, Rixensart, Belgium), according to the manufacturer's instructions.

Statistical analysis

All data are expressed as means±SEM. Comparisons between the different groups were performed by ANOVA followed by Bonferroni *t* test. A *p* value of <0.05 was accepted as statistically significant.

Results

ROS production and oxidative-stress markers in IR cells

Compared to the control cells, IR cardiac cells exhibited a significant increase in ROS cellular levels. As indicated by the DCFH-DA fluorescence, this increase started at 5 min of reoxygenation and reached a peak at 10 min; at 60 min control levels were restored (Fig. 1). After 60 min of reoxygenation, the concentration of 8-isoprostanes (typical stable end products of the lipoperoxidative process) was significantly higher in the IR than in the control cells (Fig. 2A) and concordant results were obtained using the fluorescent lipid probe BODIPY 581/591 C11 (Fig. 2B). Concomitant with the increased levels of oxidative-stress markers, TAC, assayed as an index of the antioxidant defenses, was significantly reduced (Fig. 2C). When curcumin and Trolox treatments were performed before the beginning of the ischemic phase (pretreatment) they produced significant changes in the above parameters: treated IR cardiac cells, in fact, exhibited lower increases in ROS cellular levels, reduced levels of oxidative stress markers, and a higher TAC compared to the corresponding untreated cells. On the other hand, when curcumin and Trolox were added at the moment of the reperfusion (posttreatment) they were revealed to be less effective in reducing oxidative stress markers, although the TAC levels were higher than those observed in the pretreatments.

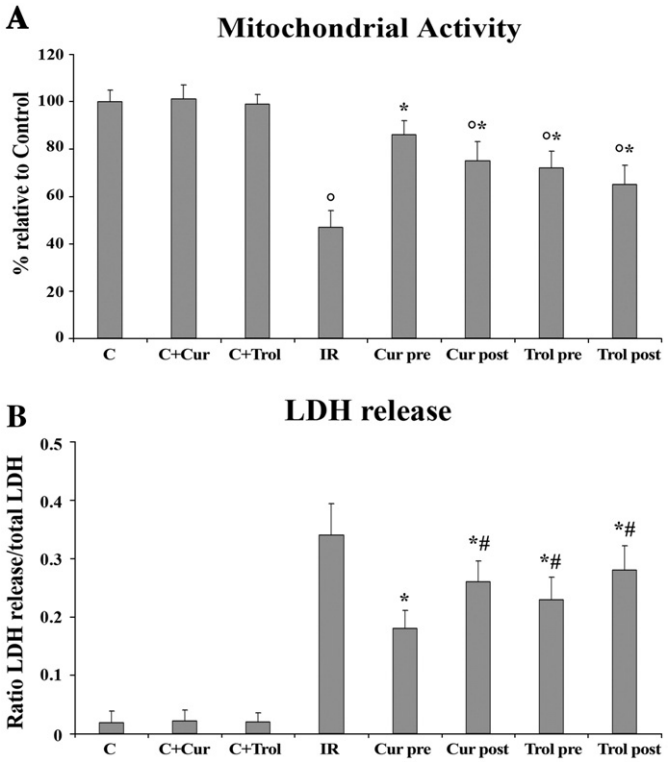

Fig. 3. Mitochondrial activity in IR H9c2 cells. (A) Mitochondrial activity was checked by the MTT test in cells exposed or not to 10 μM curcumin or 25 μM Trolox for 1 h. Values are expressed as a percentage vs control (C) cells. The reported values (means±SD) are representative of five independent experiments, each performed in triplicate. °Significant difference ($p \leq 0.05$) vs control (C) cells. *Significant difference ($p \leq 0.05$) vs IR cells. (B) The release of cellular LDH into the culture medium of IR H9c2 cells treated or not with 10 μM curcumin or 25 μM Trolox for 1 h was measured using a spectrofluorimeter. LDH release was calculated as a percentage of total LDH content. The reported values (means±SD) are representative of five independent experiments, each performed in triplicate. *Significant difference ($p \leq 0.05$) vs IR cells. #Significant difference ($p \leq 0.05$) vs curcumin-pretreated (Cur pre) cells.

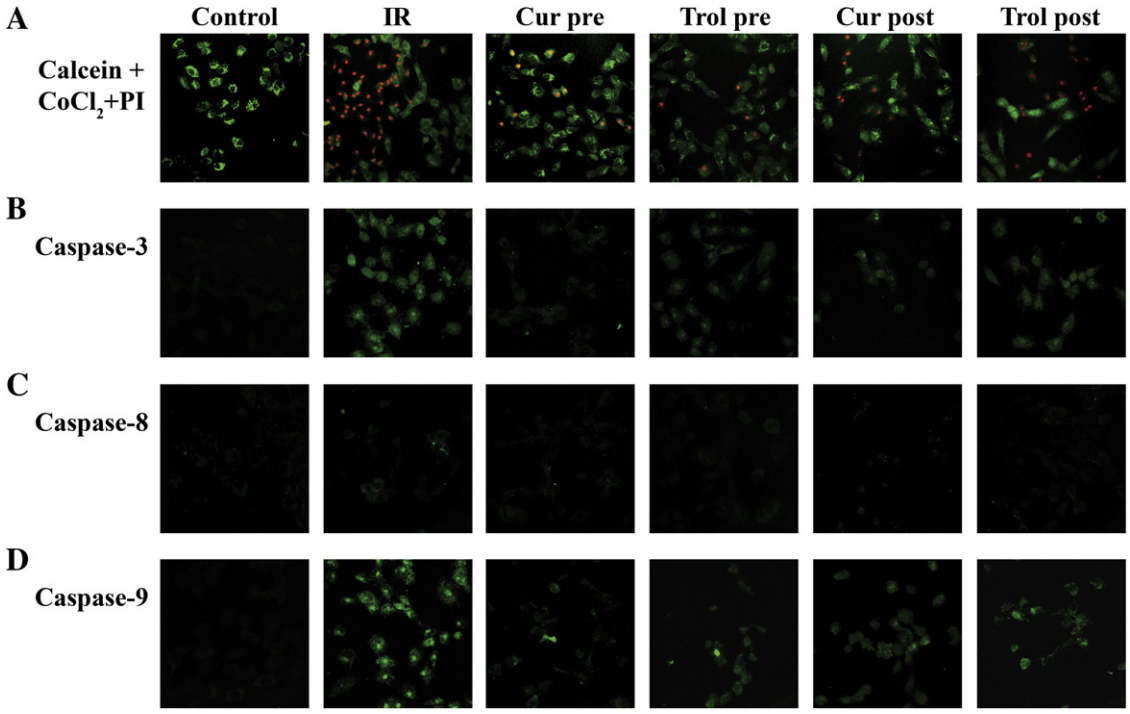

Fig. 4. Representative confocal microscopy images showing IR H9c2 cell outcome. (A) Apoptotic and necrotic cells were imaged by using calcein (green) and PI (red) as fluorescent probes, respectively. Cells were coloaded with $CoCl_2$. The activation of the apoptotic program was assessed by measuring fluorescence changes in (B) caspase-3, (C) caspase-8, and (D) caspase-9 activity. For details, see Materials and methods. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Effects on cellular viability and assessment of necrosis and apoptosis

The results of the MTT reduction test (Fig. 3A) indicate that mitochondrial activity was significantly reduced in IR cardiac cells compared to the control cells. Curcumin pretreatment almost suppressed this effect; in contrast, with the other treatments (curcumin posttreatment, Trolox pre- and posttreatment) the mitochondrial activity of IR cells, though improved, was always significantly lower compared to the control level.

LDH release in the culture medium (Fig. 3B), a finding that is considered to account for cell mortality and, especially for necrotic death, was markedly and significantly increased by the IR conditions used in the present study. LDH release was significantly reduced by all treatments; but also in this case the pretreatment with curcumin, resulting in about 50% of the level observed for the untreated IR cells, proved to be the most effective. These results agree with those provided by confocal microscopy analysis using the calcein/PI staining. As is evident from the images shown in Fig. 4A, the pretreatment with curcumin induced the most marked reduction in the number of the necrotic and apoptotic cells.

Confocal analysis was also used for a quali-quantitative assessment of the apoptotic processes, based on the changes in fluorescence associated with the activation of caspase-3, -8, and -9. The images in Figs. 4B, 4C, and 4D show a marked activation of caspase-3 and -9 in our IR cells and indicate curcumin pretreatment as the most effective in attenuating this effect.

NF-κB nuclear translocation and JNK activation: occurrence and role in the outcome of IR cells

With respect to the control cells, IR cardiac cells exhibited more than threefold higher nuclear levels of NF-κB (Fig. 5) and a significant increase in the ratio between the phosphorylated and the unphosphorylated forms of JNK (pJNK/JNK; Fig. 6). These effects, indicative of an activation of the related signaling pathways, were significantly reduced by all the treatments performed in the present study, but curcumin pretreatment was the most effective. Only this treatment, in fact, was able to reduce nuclear NF-κB and the pJNK/JNK ratio to levels that did not significantly differ from the control value and were comparable to those produced by Bay 11–7082 and SP600125 at concentrations of 10 μM, which are known to completely inhibit these processes (Figs. 7A and 7B).

Fig. 8 shows the results of experiments aimed at quantifying the roles of NF-κB and JNK in the outcome of IR cells and, at the same time, providing information about the mechanism(s) underlying the beneficial effect of curcumin. As judged by the LDH release, the combined effects of Bay 11–7082 and SP600125 significantly decreased, by about 20%, cell death in IR cells. When curcumin and Trolox pretreatments were performed in the presence of these

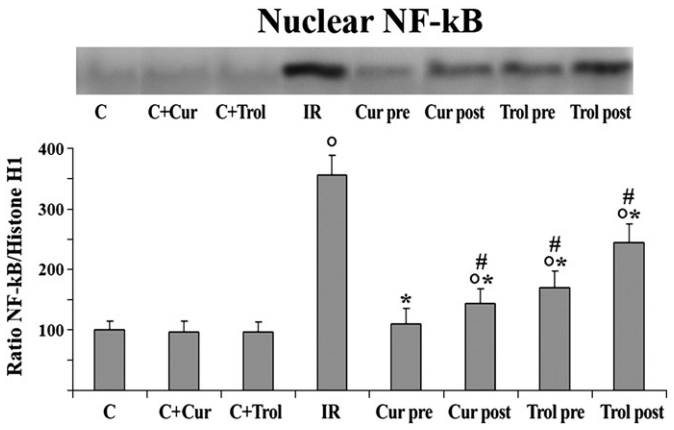

Fig. 5. Western blot analysis of NF-κB All signals were quantified by densitometric analysis and are expressed as the ratio of NF-κB densitometry to Histone H1 (loading control) densitometry. Each bar represents the mean±SEM of five different blots and relative densitometric quantifications. °Significant difference ($p \leq 0.05$) vs control (C) cells. *Significant difference ($p \leq 0.05$) vs IR cells. #Significant difference ($p \leq 0.05$) vs curcumin-pretreated (Cur pre) cells.

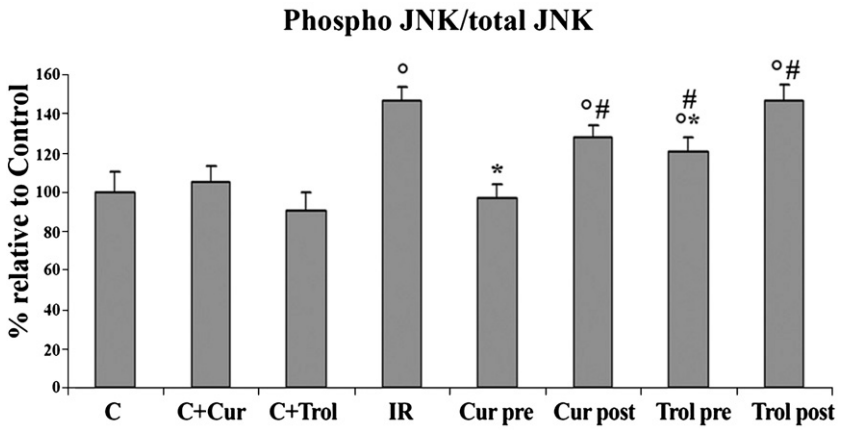

Fig. 6. Quantification of total and phosphorylated c-Jun N-terminal kinase in IR H9c2 cardiac cells. The reported values (means ± SD) are representative of five independent experiments, each performed in triplicate. °Significant difference ($p \leq 0.05$) vs control (C) cells. *Significant difference ($p \leq 0.05$) vs IR cells. #Significant difference ($p \leq 0.05$) vs curcumin-pretreated (Cur pre) cells.

inhibitors there was, in both cases, a further reduction in LDH release but this effect was less marked for curcumin treatment: as a result, no significant difference was found under these conditions between the two treatments.

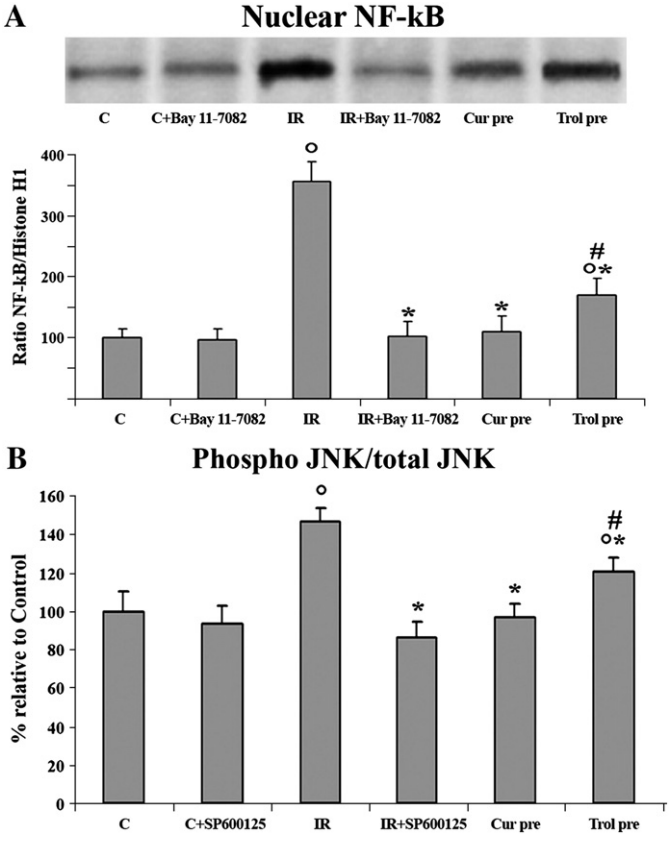

Fig. 7. (A) Western blot analysis of NF-κB activation in IR cardiac cells treated or not with 10 μM curcumin or 25 μM Trolox in the presence or absence of Bay 11–7082. All signals were quantified by densitometric analysis and are expressed as the ratio of NF-κB densitometry to histone H1 (loading control) densitometry. Each bar represents the mean ± SEM of five different blots and relative densitometric quantifications. °Significant difference ($p \leq 0.05$) vs control (C) cells. *Significant difference ($p \leq 0.05$) vs IR cells. #Significant difference ($p \leq 0.05$) vs curcumin-pretreated (Cur pre) cells. (B) Quantification of total and phosphorylated c-Jun N-terminal kinase in IR H9c2 cardiac cells treated with SP600125. The reported values (means ± SD) are representative of five independent experiments, each performed in triplicate. °Significant difference ($p \leq 0.05$) vs control (C) cells. *Significant difference ($p \leq 0.05$) vs IR cells. #Significant difference ($p \leq 0.05$) vs curcumin-pretreated (Cur pre) cells.

Effects of curcumin and Trolox on control cells

As illustrated in Figs. 2, 3, 5, and 6, curcumin and Trolox were added, at the same concentrations used for the treatments of IR cells, to the control normoxic cardiac cells to verify a possible direct effect of these substances on the considered parameters, independent of the IR conditions: no significant changes were observed in the control cells, except for an expected increase in their total antioxidant capacity. Bay 11–7082 and SP600125, when added to control cells at the same concentrations used during the experiments, revealed no toxic effects (no LDH release), as shown in Fig. 8.

Discussion

Curcumin has been found to possess a variety of pharmacological and biological activities [10], and a particular interest in this substance, in view of a potential clinical employment, comes from the fact that it is a nontoxic and nonmutagenic natural product. It has been recently demonstrated that a 1–15 μM concentration of curcumin does not cause cytotoxicity [21]. The use of curcumin as a therapeutic agent has been investigated in a number of pathological conditions, including cardiovascular diseases and other vascular dysfunctions [22,23]. Several recent reports also indicate that curcumin can attenuate the damages induced by postischemic reperfusion in various tissues and under various experimental

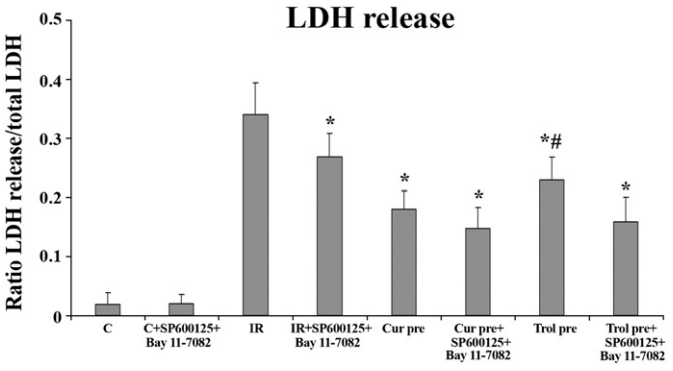

Fig. 8. Release of cellular LDH into the culture medium of IR H9c2 cells treated or not with 10 μM curcumin or 25 μM Trolox in the presence or absence of Bay 11–7082 and SP600125. LDH release was calculated as a percentage of total LDH content. The reported values (means ± SD) are representative of five independent experiments, each performed in triplicate. *Significant difference ($p \leq 0.05$) vs IR cells. #Significant difference ($p \leq 0.05$) vs curcumin-pretreated (Cur pre) cells.

conditions [24–26]. To a great extent, such effects are ascribed to the strong antioxidant activity of curcumin [27], a substance that we found (data not shown) to have a free radical scavenging activity more then twice as high with respect to Trolox, a compound commonly used as a standard in this kind of measurement.

To verify the effectiveness of curcumin in protecting cardiac cells against IR injury we examined the effects of two different treatments with this substance (the above-described pre- and posttreatment) on H9c2 cardiac cells subjected to a well-established protocol of simulated IR. H9c2 cells are extensively used in cardiologic research because they have been found to possess elements and properties of signaling pathways of the adult myocyte [28]; thus we thought that this was a suitable model for the purposes of this study. In both treatments the final curcumin concentration in the culture medium was 10 μM, a level that was confirmed to be devoid of cytotoxicity by our results and did not induce significant changes in the MTT test nor in LDH release with respect to the untreated control cells. Ten micromolar is a concentration frequently used for curcumin in studies with cultured cells and it is on the same order of magnitude as the levels that may be achieved from dietary intake. According to a recent report on this subject [29] a concentration of 3.6 μM is reached in human serum with a curcumin oral administration of 8 g/day, but this compound is safe even at higher doses (12 g/day) and its bioavailability may be improved by several approaches, such as the use of an adjuvant like piperine or the use of liposomal curcumin or curcumin nanoparticles.

Our measurements were intended to establish: (i) the occurrence and the intensity of oxidative stress; (ii) the effects on cellular viability, including the assessment of necrotic and apoptotic cell death; and (iii) a possible involvement of the signaling pathways related to NF-κB and JNK, because their activation in response to oxidative stress may result in cell apoptosis/necrosis. To see whether the effects of curcumin were to be ascribed only to its antioxidant activity, all the above determinations were performed in IR cardiac cells subjected to the same pre- and posttreatments with 25 μM Trolox, the concentration required to obtain the same antioxidant capacity of 10 μM curcumin. In addition, treatments with Bay 11–7082 and SP600125, used at proper concentrations, were performed to explore, even from a quantitative point of view, the roles of NF-κB and JNK in the outcome of IR cells and the effects of curcumin and Trolox on these pathways.

From the obtained results it seems that the IR conditions realized in the present study were associated with a patent oxidative stress and resulted in impaired cell viability, as judged by the MTT reduction test. At the same time, the increase in the ratio of released to total LDH, which was about 10-fold higher with respect to the control values, indicated a considerable development of the cell death processes. As for the mode of cell death, the occurrence of apoptosis, in addition to necrosis, was evident from the results of confocal microscopy analysis that we performed to assess the outcome of IR cells and caspase activation. Finally, the observed increases in NF-κB nuclear translocation and in JNK phosphorylation suggest a significant activation of the pathways related to these factors and their potential role in the damages affecting our IR cardiac cells.

A comparative valuation of the various treatments performed in this study clearly shows that both curcumin and Trolox pretreatments were more effective than the corresponding posttreatments in attenuating oxidative stress and in limiting cell injury. This finding is probably ascribable to the fact that in the posttreatments the intracellular levels of curcumin and Trolox, despite the quick cellular absorption of both these compounds, were not high enough to protect against the burst of ROS production that, as stated above, occurred after a few minutes of simulated reperfusion. According to this interpretation, the apparently contradictory result of a higher total antioxidant capacity in the posttreated than in the pretreated cells may simply reflect a reduced consumption of the added antioxidants.

If we restrict the comparison to the pretreatments, it is evident that, although curcumin and Trolox were used at concentrations with the same scavenging activity and their addition produced similar levels of total antioxidant capacity in exposed cells, the protective effects of the two treatments were quite different. Curcumin, in fact, was significantly more effective than Trolox in preserving the viability of IR cardiac cells and in reducing total and, in particular apoptotic, cell death. Taken together, these results suggest that curcumin can provide a substantial protection against IR injury, not only through its antioxidant properties but also via other mechanisms, acting at the biochemical and/or the cellular level. In this context we found that both NF-κB translocation to the nucleus and JNK phosphorylation, which are crucial steps in the activation of the pathways involving these factors, were markedly reduced by curcumin pretreatment of IR cardiac cells. Other investigators, using different experimental models, have reported that curcumin inhibits NF-κB- and JNK-related pathways and protects cells experiencing oxidative stress or other stressing agents [30,31]. The novel finding arising from the present study is that these actions are also partly attributable to the antioxidant properties of curcumin, because the pretreatment of IR cells with this compound resulted in an inhibition of NF-κB nuclear translocation and of JNK phosphorylation that was quantitatively comparable to that observed, respectively, for Bay 11–7082 and SP600125 and significantly higher than that induced by Trolox.

This leads us to suppose a direct molecular interaction of curcumin in the above-mentioned pathways, an interpretation in line with several, although fragmentary, reports on this subject. Indeed, it has been reported that curcumin can inhibit IKK [32–34], an essential enzyme for NF-κB activation, and can induce covalent modifications affecting NF-κB binding to DNA [35,36]. As for JNK, it has been found that curcumin may interfere at the proximal upstream portion of this pathway, in particular at the MAPK kinase kinase level [31]. Moreover, in light of recent findings indicating a cross talk between NF-κB and JNK [37], the above-mentioned effects might be independent or reciprocally related.

In this study a strong indication of the possibility of a direct interaction of curcumin in the NF-κB and JNK pathways, and of a link between this event and the protective effect of the agent, was provided by the comparison of the changes in LDH release observed in the IR cells pretreated with curcumin and Trolox with those produced by similar pretreatments with Bay 11–7082 and SP600125.

On one hand, the significant reduction in LDH release obtained with the two inhibitors confirms that the activation of the NF-κB and JNK pathways has an important role, even in our model, in the outcome of the IR cells. On the other hand, the absence of a significant difference in LDH release between curcumin and Trolox pretreatments when they were performed in the presence of Bay 11–7082 and SP600125, that is, under conditions precluding further effects on NF-κB and JNK, leads us to believe that the higher protection exhibited by curcumin treatment is due to the greater extent to which it can affect these pathways, an effect that implies other interactions in addition to the antioxidant power because, as repeatedly stated, the two compounds were used at a parity of antioxidant capacity.

In conclusion, the results of this study indicate that a shrewd use of curcumin may represent a promising approach to protecting cardiac cells against IR injury. In this scenario our findings point out the importance of the chronology for the treatment outcome and represent the first attempt (at least to our knowledge) to differentiate the degree of protection that curcumin can exert by its antioxidant activity and by other mechanisms. To better understand the usefulness of curcumin in protecting heart from ischemia-reperfusion injury, our results support the exploration of the effects of this compound on cardiac cells other than myocytes, a subject of only a few reports [38,39] in the current literature, and the study of the feasible molecular interactions with NF-κB, JNK, and other oxidative stress-dependent pathways potentially involved in apoptotic/necrotic

cell death. Interesting information might be so obtained for adjusting and/or integrating curcumin treatment to optimize the protective effect, whereas in vivo studies will confirm the reaching of this goal.

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