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Curcumin reduces cold storage-induced damage in human cardiac myoblasts

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Abbreviations: HO-1, heme oxygenase-1; LDH, lactate dehydrogenase; SnPPIX, tin protoporphyrin IX

Abstract

Curcumin is a polyphenolic compound possessing interesting anti-inflammatory and antioxidant properties and has the ability to induce the defensive protein heme oxygenase-1 (HO-1). The objective of this study was to investigate whether curcumin protects against cold storage-mediated damage of human adult atrial myoblast cells (Girardi cells) and to assess the potential involvement of HO-1 in this process. Girardi cells were exposed to either normothermic or hypothermic conditions in Celsior preservation solution in the presence or absence of curcumin. HO-1 protein expression and heme oxygenase activity as well as cellular damage were assessed after cold storage or cold storage followed by re-warming. In additional experiments, an inhibitor of heme oxygenase activity (tin protoporphyrin IX, 10 μM) or siRNA for HO-1 were used to investigate the participation of HO-1 as a mediator of curcumin- induced effects. Treatment with curcumin produced a marked induction of cardiac HO-1 in normothermic condition but cells were less responsive to the polyphenolic compound at low temperature. Cold storage-induced damage was markedly reduced in the presence of curcumin and HO-1 contributed to some extent to this effect. Thus, curcumin added to Celsior preservation solution effectively prevents the damage caused by cold-storage; this effect involves the protective enzyme HO-1 but also other not yet identified mechanisms.

Keywords: cryopreservation curcumin; cytoprotection; heme oxygenase-1; organ preservation; organ transplantation; oxidative stress

Introduction

Curcumin, the yellow pigment derived from turmeric, has been investigated for some time because of its interesting antioxidant, anti-inflammatory and anti-carcinogenic activities (Ammon and Wahl, 1991). In particular, curcumin belongs to the family of polyphenolic compounds which modulate the activities of the pro-inflammatory enzymes cyclooxygenase and inducible nitric oxide synthase (Bengmark, 2006), while at the same time inducing the gene expression of phase II detoxification enzymes involved in protection against carcinogenesis (Dinkova-Kostova, 2002). The action of curcumin appears to stimulate the activation of NrF2, a transcriptional factor responsible for the up-regulation of antioxidant and protective proteins (Balogun *et al.,* 2003b). Our group has investigated the ability of curcumin to induce the stress protein heme oxygenase-1 (HO-1) in a variety of cell types and reported on the increased expression of HO-1 by curcumin via regulation of NrF2 and the antioxidant response element (Motterlini *et al.,* 2000b; Scapagnini *et al.,* 2002; Balogun *et al.,* 2003b). In addition, we have shown that the antioxidant activity of curcumin is partially mediated by HO-1 induction in normal culture conditions and also following incubation of cells at low temperatures (Balogun *et al.,* 2003a). This last aspect is of special interest for transplantation procedures as organs are usually kept at 4°C in storage solutions before the actual transplant surgery. HO-1 and its products biliverdin, bilirubin and carbon monoxide exert beneficial effects that include protection against ischemia-reperfusion damage (Clark *et al.,* 2000b, 2003; Foresti *et al.,* 2001; Sandouka *et al.,* 2006), mitigation of oxidative and nitrosative stress (Clark *et al.,* 2000a; Kaur *et al.,* 2003; Taille *et al.,* 2005) and prolongation of graft survival of liver, heart and kidney allografts in experimental animal models of transplantation (Soares *et al.,* 2001; Clark *et al.,* 2003; Motterlini *et al.,* 2005; Nakao *et al.,* 2005). Because of its antioxidant and anti-inflammatory characteristics, the HO-1 pathway is potentially an excellent candidate to be exploited for amelioration of organ transplantation outcomes and survival (Motterlini *et al.,* 2003, 2005; Otterbein

et al., 2003; Sandouka *et al.,* 2006). An ideal clinical situation could rely on agents/conditions that induce HO-1 during organ storage and subsequently maintain its expression elevated for the transplant surgery. This is based on the idea that cold storage itself, despite slowing cellular metabolism and extending cellular viability, also results in cold-associated injury caused by reactive oxygen species (Ahlenstiel *et al.,* 2006). For this reason, special cold storage solutions have been developed with the aim to preserve organ function and viability. They usually contain high potassium to prevent loss of potassium-ATPase activity and include a colloid to reduce cell swelling (McLaren and Friend*,* 2003). One such solution, named Celsior, has been recently introduced in heart transplantation with the aim of combining general principles of hypothermic storage with the specific needs of the myocardium (Michel *et al.,* 2002). In comparison to other storage solutions, Celsior seems to better preserve left ventricular functions and maintain energy levels (Michel *et al.,* 2002). In addition, Celsior has the further advantage of being used uniformly as a perfusion, storage and post-storage perfusion solution.

In view of the beneficial effects elicited by curcumin and HO-1 against oxidative stress and inflammation, we analyzed in the present study whether addition of curcumin to Celsior could enhance the ability of the solution to protect against cold storage-mediated damage of cultured human cardiomyocytes. We also assessed the potential role of HO-1 in the phenomenon observed.

Materials and Methods

Reagents

Hemin and tin protoporphyrin IX (SnPPIX) were obtained from Porphyrin Products INC (Logan, Utah). Celsior solution was purchased from IMTIX SANGSTAT. The composition of Celsior solution was the following: 60 mM mannitol, 80 mM lactobionic acid, 20 mM glutamic acid, 100 mM sodium hydroxide, 0.25 mM calcium chloride, 15 mM potassium chloride, 13 mM magnesium chloride, 30 mM histidine and 3 mM glutathione. Curcumin and all other reagents were purchased from Sigma unless otherwise stated. Solutions of hemin, SnPPIX and curcumin were prepared freshly before each experiment.

Cell culture and experimental protocol

Human adult atrial myoblast cells (Girardi cells) were purchased from the European Collection of Animal Cell Culture (Salisbury, UK) and cultured in DMEM supplemented with 1% non essential amino acids, 10% FBS and 3.5 mM L-glutamine. Cells were grown in 75-cm^2 flasks and maintained at 37°C in a humidified atmosphere of air (5% CO_2). For experiments conducted in normothermia, cells were exposed to curcumin (0-30 μM) or hemin (0-20 μM) in culture medium for different times and heme oxygenase activity and HO-1 expression were determined at the end of the incubation. Cells pre-incubated with curcumin for 6 h were challenged with hydrogen peroxide (3 and 6 mM) for an additional 6 h and cell viability assessed using Alamar Blue and LDH release assays. In some experiments SnPPIX (10 μM), an inhibitor of heme oxygenase activity, was added to determine the potential involvement of HO-1. For studies conducted under hypothermic conditions, heme oxygenase levels were measured in cells treated with 15 μM curcumin in Celsior solution under a programmed change in temperature consisting of 3 h incubation at 37°C followed by an additional 3 h at 4°C. In some experiments hemin (5 μM) was co-incubated with curcumin. The effect of the programmed change in temperature on cell metabolism and cell viability was assessed using the Alamar Blue assay, lactate dehydrogenase (LDH) release and trypan blue exclusion methods. In a different set of experiments the damage caused by cold storage (6 h at 4°C in Celsior solution) was measured in cardiomyocytes in the presence or absence of curcumin (15 μM). Viability was also determined in cells subjected to cold storage in Celsior solution for 6 h and re-warmed for 1 h at 37°C in culture medium. To investigate the involvement of HO-1, SnPPIX (10 μM) was used under the same experimental conditions. Finally, cells were incubated in cold storage for different times (4-18 h) in the presence or absence of curcumin and cytotoxicity measured at the end of the experiment by LDH release. To address the contribution of HO-1, a parallel set of experiments was performed using siRNA for HO-1.

Heme oxygenase activity assay

Heme oxygenase activity was determined at the end of each treatment as described previously by our group (Motterlini *et al.,* 1996). Briefly, harvested cells were added to a reaction mixture containing NADPH, glucose-6-phosphate dehydrogenase, rat liver cytosol as a source of biliverdin reductase, and the substrate hemin. At the end of the reaction bilirubin was extracted by the addition of 1 ml of chloroform and was measured by the difference in absorbance between 464 and 530 nm (ε = 40 $mM^{-1}cm^{-1}$).

Western blot analysis for HO-1

Western immunoblot technique was performed by separating an equal amount of proteins (30 μg) for each sample by SDS-PAGE, transferred overnight to nitrocellulose membranes and the non-specific binding of antibodies was blocked with 3% non-fat dried milk in PBS as described (Motterlini *et al.,* 2000a).

RNA isolation and PCR

RNA isolation was performed using Purescript® RNA Isolation Kit (Gentra, Minneapolis, MN) according to the manufacturers' instruction. Total RNA (1 μg) was reverse transcribed using random hexamer primers (GeneAmp RNA PCR kit, Perkin-Elmer, Norwalk, CT). With the use of a manual hot start, the cDNA was amplified using 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer), with the 3' and 5' primers for both GAPDH and HO-1 being added to the same reaction tube (these yielded products of 247 and 583 bp, respectively). After an initial melting at 95°C, the PCR mixtures were amplified using a UNO II thermocycler (Biometra, Göttingen, Germany) for a total of 34 cycles, using a two-step protocol of melting at 95°C for 1 min and annealing at 61°C for 1.5 min. The product (20 μl/ lane) was run on a 2.5% agarose gel (Metaphor agarose, FMC Bioproducts, Rockland, ME).

Cell viability assays

The effect of various treatments on Girardi cells viability was determined using different assays. An Alamar Blue assay kit (Serotec, U.K.) was used according to manufacturer's instructions as previously reported (Motterlini *et al.,* 2000b). This spectrophotometric assay is based on the detection of metabolic activity of living cells using a redox indicator. The release of lactate dehydrogenase (LDH) as an index of cell damage was also measured in the culture supernatant using a cytotoxicity detection kit (Roche) following manufacturers' instructions. The LDH released was expressed as percentage of total LDH activity determined following lysis of cells with Triton X-100 (1% in DMEM at 25°C). The number of dead cells was assessed using the trypan blue exclusion method. Briefly, cells at the end of the treatment were trypsinized and centrifuged (5 min at 500 rpm), followed by resuspension with trypan blue solution (0.4% in PBS, 1:1 dilution with culture medium) for ~2 min. The number of dead cells (retaining the dye) was counted with a hemocytometer and expressed as a percentage of the total cell number.

Transfection of Girardi cells with small interfering RNA (siRNA) for HO-1

Girardi cells were grown in twelve-well plates and transiently transfected with HO-1 siRNA mixed with the appropriate transfection reagent (Santa Cruz Biotechnology) according to the manufacturer's instructions and as previously reported by us (Abuarqoub *et al.,* 2006). After incubation at 37°C for 30 h, cells were exposed to hypothermia for 4, 6, 8, 12, and 24 h in the presence or absence of curcumin (15 μM) as described in the Experimental Protocol. Samples were then collected and analyzed for LDH release.

Statistical analysis

Differences among the groups were analyzed using one-way analysis of variance combined with the Bonferroni test. Values were expressed as mean ± S.E.M. and differences between groups were considered to be significant at $P < 0.05$.

Results

Curcumin and hemin increase HO-1 protein in human cardiomyocytes incubated under normothermic conditions

We initially tested the response of Girardi cells to common inducers of HO-1 in normothermia. As shown in Figure1, heme oxygenase activity and HO-1 expression were elevated following incubation of cells for 6 h with increasing concentrations of either hemin (Figure 1A) or curcumin (Figure 1B). Heme oxygenase activity was also increased over time by curcumin (Figure 1C) with a pattern similar to the one reported for other cell types (Motterlini *et al.,* 2000b; Scapagnini *et al.,* 2002). When challenged with hydrogen peroxide, Girardi cells exhibited a decrease in cell viability (Figure 2A), an effect that was completely abolished by co-incubation with curcumin. Interestingly, hydrogen peroxide caused a significant loss of viability when cells were exposed to curcumin in the presence of the heme oxygenase inhibitor SnPPIX, suggesting that heme oxygenase activity was involved in the protection elicited by curcumin in normothermic conditions. These data are in line with previous results using aortic endothelial cells (Motterlini *et al.,* 2000b). A similar trend was observed when LDH release by cells was measured. In fact, curcumin decreased the LDH release induced by 3 and 6 mM hydrogen peroxide (Figure 2B).

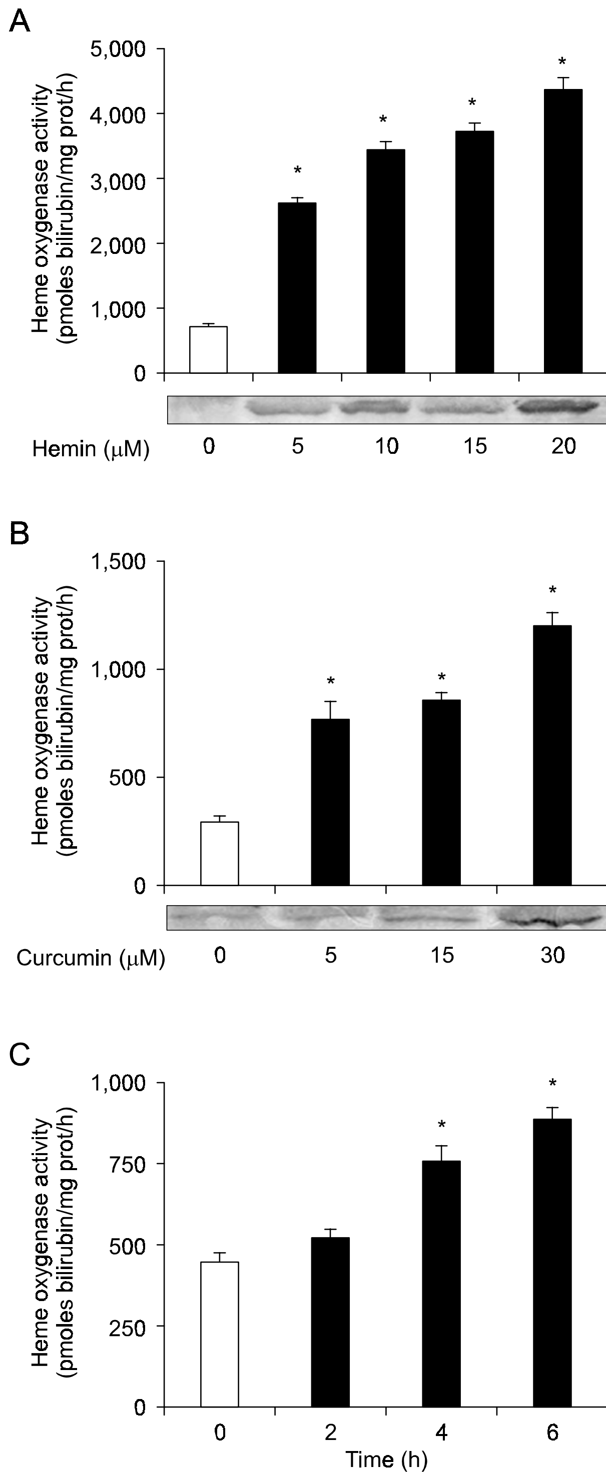

Figure 1. Curcumin and hemin induce HO-1 in human cardiomyocytes under normothermic conditions. Heme oxygenase activity and HO-1 expression were determined in Girardi cells 6 h after exposure to hemin (0-20 μM) (A) or curcumin (0-30 μM) (B). Heme oxygenase activity was also assessed after incubation of cells with 15 μM curcumin for 0, 2, 4 or 6 h (C). *$P < 0.05$ *vs.* control (open bars).

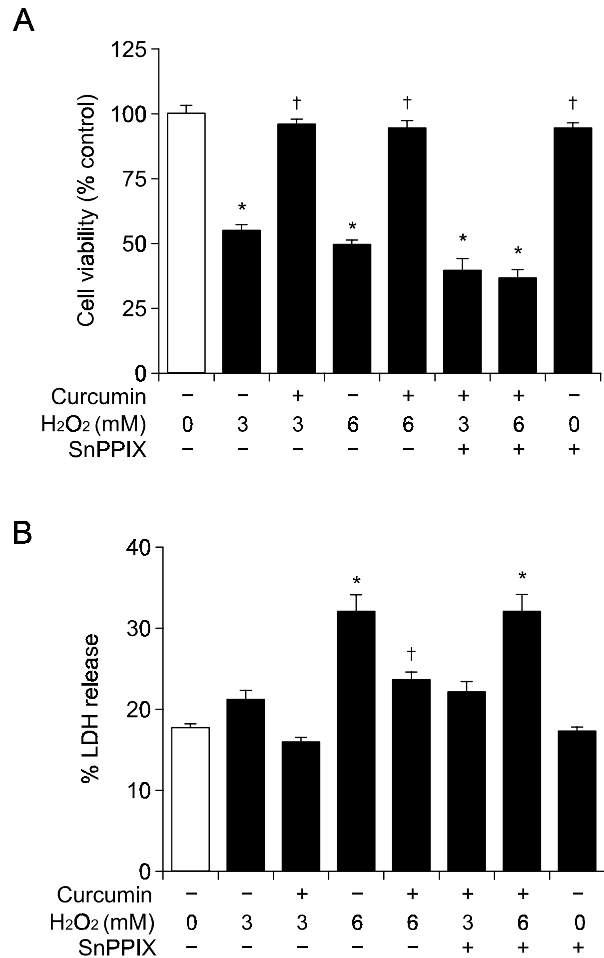

Figure 2. Curcumin protects against hydrogen peroxide-mediated cellular damage. Cell viability (A) and LDH release (B) were determined after 6 h exposure to hydrogen peroxide (H_2O_2, 3 or 6 mM) in untreated cells or cells pre-treated for 6 h with curcumin (15 μM) in DMEM. In some experiments, tin protoporphyrin IX (SnPPIX, 10 μM) was added during the pre-incubation time to test the potential involvement of heme oxygenase in cytoprotection. *$P < 0.05$ *vs.* untreated, †$P < 0.05$ *vs.* H_2O_2 alone.

Effect of curcumin and hemin on heme oxygenase activity and HO-1 expression in human cardiomyocytes incubated under hypothermic conditions

Cells incubated with curcumin under hypothermic conditions in Celsior solution (3 h at 37°C followed by 3 h at 4°C) showed a slight but significant ($P <$ 0.05) increase in the levels of heme oxygenase activity (Figure 3A). Under these conditions, heme oxygenase activation was higher when cells were exposed to hemin or curcumin in combination with hemin, as shown by increased expression of HO-1 mRNA (Figure 3B). In general, the response of cells to the HO-1 inducing agents was markedly lower in hypothermia compared to normothermia. This was

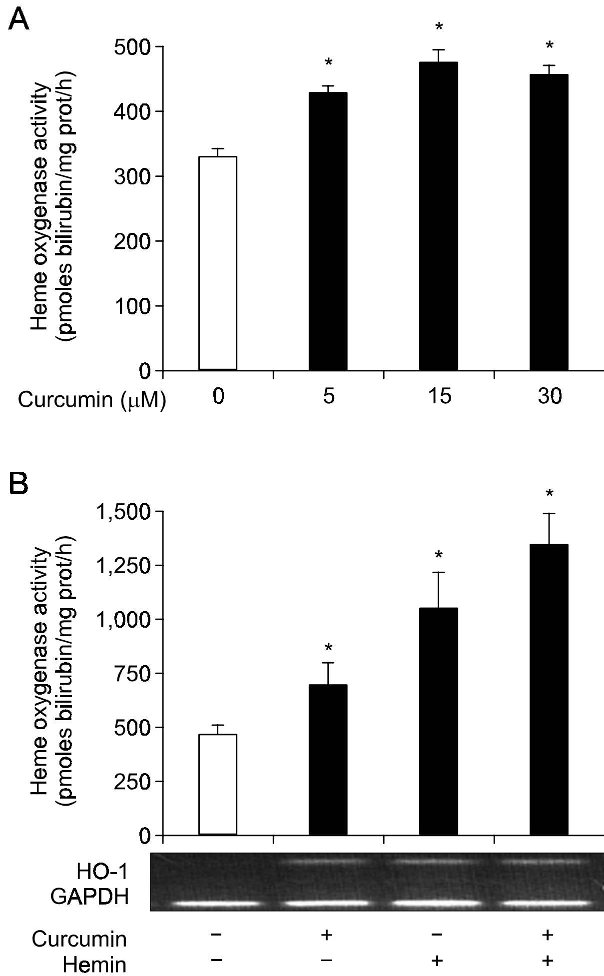

Figure 3. Heme oxygenase activity and HO-1 mRNA expression in cells exposed to curcumin and hemin under hypothermic conditions. Cells were exposed to curcumin (0-30 μM) (A) or curcumin plus hemin (5 μM) (B) dissolved in Celsior solution under hypothermic conditions. These consisted of a programmed change in temperature of 3 h incubation at 37°C followed by 3 h at 4°C. Heme oxygenase activity and HO-1 mRNA expression were measured at the end of the incubation. *$P < 0.05$ *vs.* untreated.

not unexpected and has been observed previously with other cell types (Balogun *et al.,* 2003a). Notably, experiments conducted by exposing Ghirardi cells to hypothermic conditions (4°C) in culture medium (DMEM) showed that cardiomyocytes suffered extensive cellular damage (data not shown). This was predictable since culture media are not designed to preserve organs and tissues at low temperatures and, as a consequence, storage solutions have been developed to overcome this problem. The viability of Girardi cells (Figure 4A) and the LDH release profile (Figure 4B) did not change significantly following exposure to the hypothermic protocol in Celsior solution, either in the presence or absence of curcumin. In

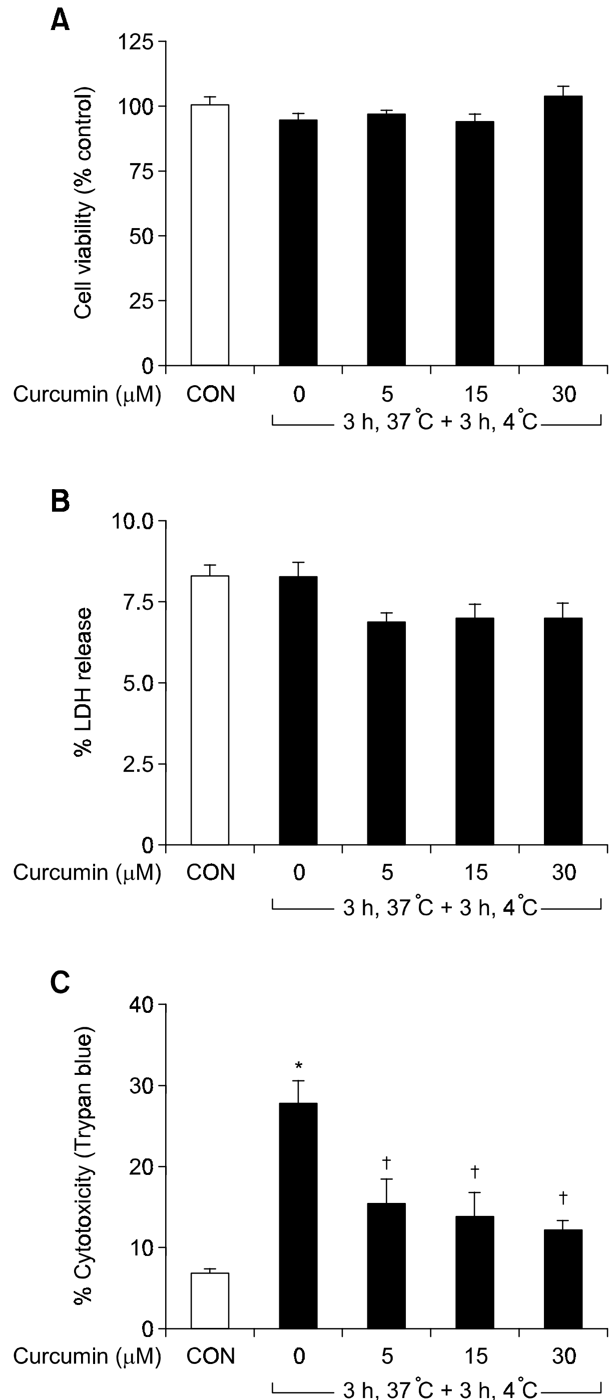

Figure 4. Effect of curcumin on cell viability, LDH release and cytotoxicity under hypothermic storage. Girardi cells were incubated for 3 h at 37°C followed by 3 h at 4°C in Celsior solution in the presence or absence of curcumin (0-30 μM). Cell viability was measured with the Alamar blue assay (see Materials and Methods) (A). LDH release was assessed in the culture medium (B) and cytotoxicity was measured by the trypan blue exclusion assay (C). The white bar represents cells exposed for 6 h to normothermia in DMEM to provide an internal control. *$P < 0.05$ *vs.* cells incubated in normothermia (white bar); $^{\dagger}P < 0.05$ *vs.* cells incubated in the absence of curcumin.

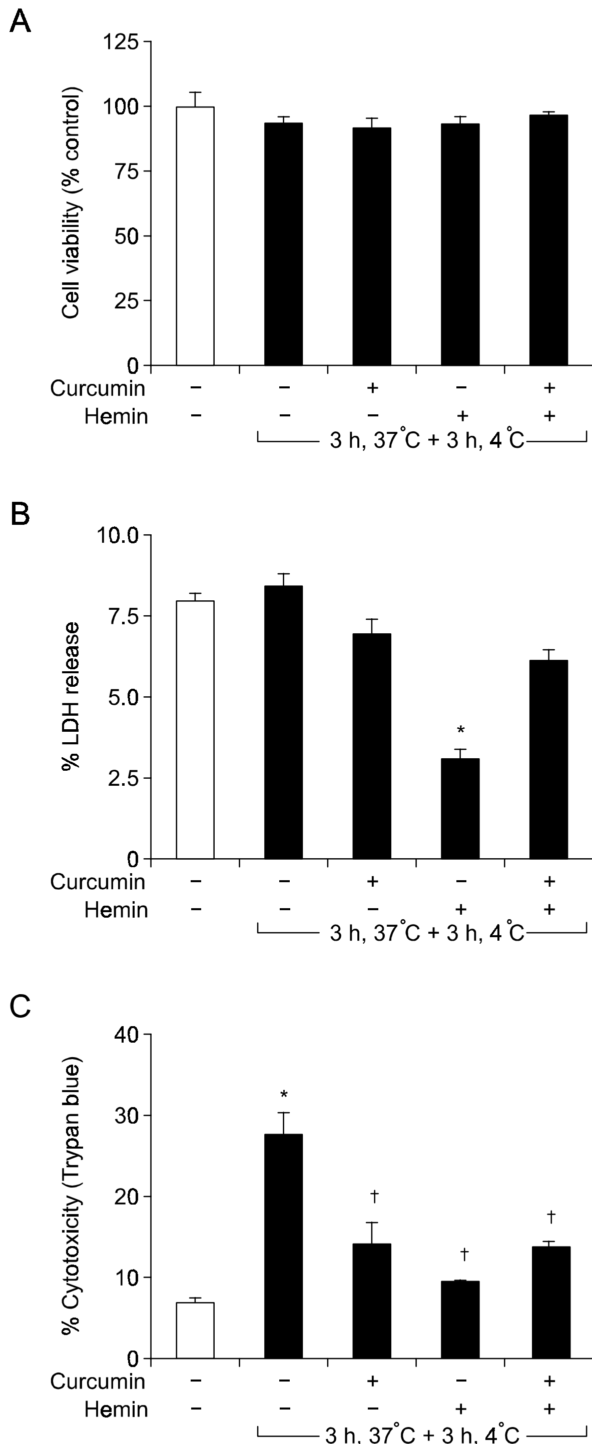

Figure 5. Effect of the combination of curcumin and hemin on cell viability, LDH release and cytotoxicity under hypothermic storage. Girardi cells were incubated for 3 h at 37°C followed by 3 h at 4°C in Celsior solution in the presence or absence of curcumin (15 μM) alone or in combination with hemin (5 μM). Cell viability was measured with the Alamar blue assay (A). LDH release was assessed in the culture medium (B) and cytotoxicity was measured by trypan blue exclusion assay (C). The white bar represents the results obtained in cells exposed for 6 h to normothermia in DMEM to provide an internal control. *$P < 0.05$ *vs.* cells incubated in normothermia (white bar); † $P < 0.05$ *vs.* cells incubated in the absence of curcumin plus hemin.

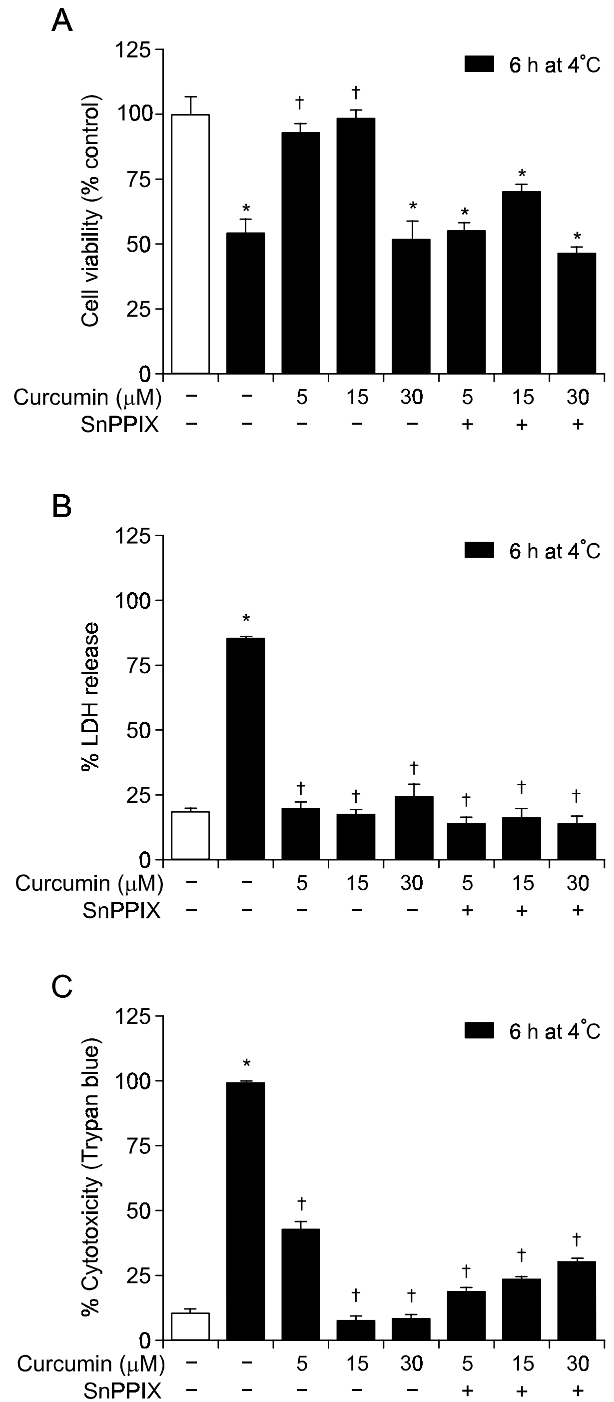

Figure 6. Curcumin protects against cellular damage caused by prolonged cold storage in Celsior solution. Cells were exposed to cold storage in Celsior solution for 6 h at 4°C in the presence or absence of curcumin (0-30 μM). In some experiments the inhibitor of heme oxygenase activity, tin protoporphyrin IX (SnPIXX, 10 μM) was also added during cold storage. Cell viability was measured with the Alamar blue assay (A). LDH release was assessed in the culture medium (B) and cytotoxicity was measured by trypan blue exclusion assay (C). The white bar represents the results obtained in cells exposed for 6 h to normothermia in DMEM to provide an internal control. *$P < 0.05$ *vs.* cells incubated in normothermia (white bar); †$P < 0.05$ *vs.* 6 h at 4°C; ‡$P < 0.05$ *vs.* 4°C + curcumin.

contrast, an increase in damage was detected in cells after hypothermic treatment using the trypan blue cytotoxicity assay; consistent with the results obtained after challenge with H_2O_2, different concentrations of curcumin markedly reduced this effect (Figure 4C). As shown in Figure 5A, co-incubation of cells with curcumin or hemin, or the combination of the two under hypothermic conditions did not change cell viability. The release of LDH was considerably decreased in the presence of hemin alone (Figure 5B); in addition, the trypan blue assay indicated that curcumin or hemin alone or the combination of these two HO-1 inducers significantly reversed the toxicity caused by hypothermia (Figure 5C).

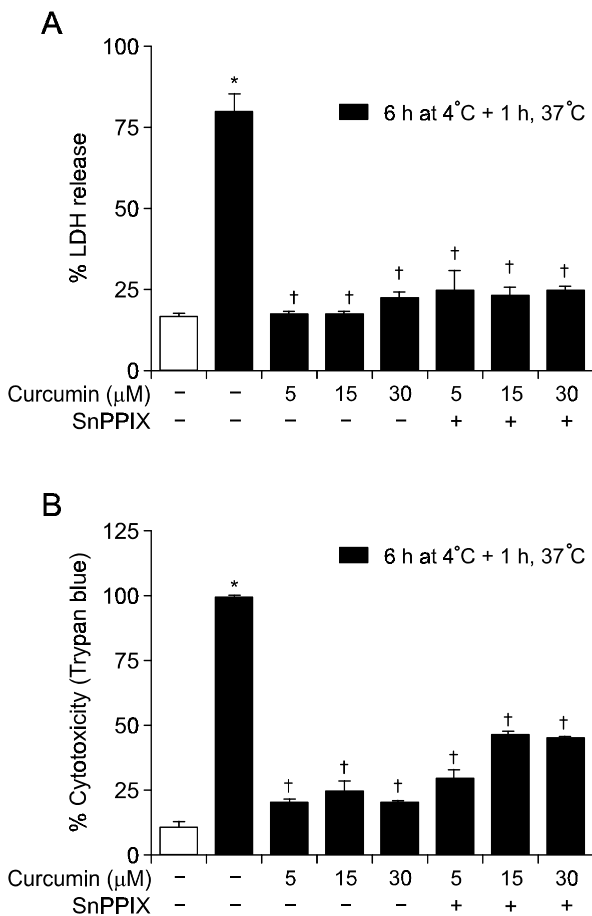

Figure 7. Cytotoxicity profile in cells exposed to cold storage followed by re-warming. Girardi cells were incubated for 6 h at 4°C in Celsior solution in the presence of curcumin alone (0-30 μM) or in combination with tin protoporphyrin IX (SnPPIX, 10 μM) and then re-warmed for 1 h at 37°C in culture medium. LDH release (A) and cytotoxicity by trypan blue exclusion assay (B) were measured. $^{*}P < 0.05$ *vs.* cells incubated in normothermia in DMEM (white bar); $^{\dagger}P < 0.05$ *vs.* 6 h at 4°C + 1 h, 37°C; $^{\ddagger}P < 0.05$ *vs.* 6 h at 4°C + 1 h, 37°C + curcumin.

Viability of Girardi cells exposed to cold storage alone or cold storage followed by re-warming

Cell viability was significantly decreased after cold storage (6 h at 4°C in Celsior solution) (Figure 6A) and this effect was accompanied by an increase in LDH release (Figure 6B) as well as cell death as measured by the trypan blue exclusion method (Figure 6C). Interestingly, 5 and 15 μM curcumin completely restored cell viability and effectively reduced LDH release and cell death (Figure 6). The protection elicited by curcumin was partially lost in the presence of SnPPIX, although this effect was not visible in terms of LDH release. Therefore, it appears that curcumin is a protective agent against cold storage damage and that heme oxygenase contributes to some extent to this effect. To better reproduce the *in vivo* situation where organs or tissues are maintained in cold storage and then re-warmed during transplantation, we also assessed the damage caused by 6 h cold storage in Celsior solution followed by 1 h re-warming in culture medium at 37°C. As shown in Figure 7A, this treatment elicited considerable LDH release but was readily prevented in the presence of curcumin. Similarly, cell death caused by cold storage and re-warming was reduced to control levels when cells were incubated with curcumin (Figure 7B). SnPPIX slightly reversed this effect (Figure 7A and 7B), suggesting again a small contribution of heme oxygenase to curcumin-mediated protection. As a final approach, the release

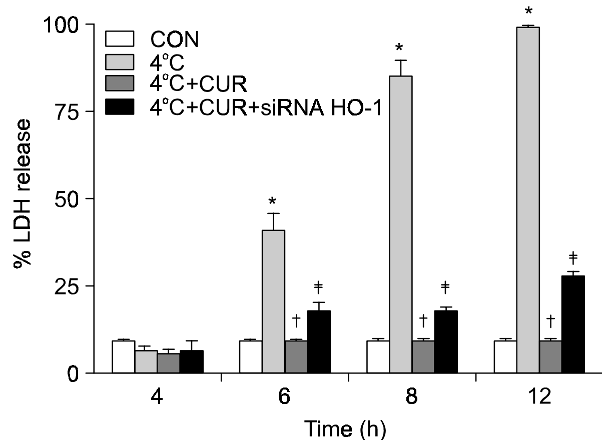

Figure 8. Effect of curcumin-mediated HO-1 induction on cytoprotection in Girardi cells. Cells were exposed to cold storage (4°C) in Celsior solution for 4, 6, 8 and 12 h in the presence or absence of 15 μM curcumin alone or in combination with siRNA for HO-1. Cell viability was measured by LDH release at the end of the incubation periods. The control group (CON) was represented by cells incubated in culture medium alone under normothermic conditions (37°C) for 12 h. $^{*}P < 0.05$ *vs.* CON; $^{\dagger}P < 0.05$ *vs.* 4°C; $^{\ddagger}P < 0.05$ *vs.* 4°C + curcumin (CUR).

of LDH was measured in Girardi cells subjected to cold storage for different times in the presence or absence of curcumin, and in cells incubated with curcumin and siRNA for HO-1. Figure 8 illustrates that cold storage produced a time-dependent damage, which was maximum at 12 h (the degree of damage at 18 and 24 h was similar, data not shown); notably, the cold storage-mediated damage was significantly decreased by the presence of curcumin. Consistent with the results obtained with SnPPIX, siRNA for HO-1 partially but significantly reversed the beneficial action of curcumin, especially at later time points.

Discussion

The present study shows that curcumin is a potent antioxidant molecule that efficiently protects human myoblasts against oxidative stress and cold storage-rewarming damage both at normal and low temperature conditions. The beneficial effects of curcumin were partially associated with its ability to induce the stress protein HO-1 but also to other properties of the molecule, which may include intrinsic antioxidant capacities and, potentially, regulation of the expression of other protective proteins. Thus, it appears that curcumin could be a good candidate to investigate as a supplement for the improvement of organ storage solutions.

In normal conditions, Girardi cells were shown to be highly responsive to inducers of HO-1 while incubation in hypothermia allowed only a small up-regulation of HO-1. It is conceivable that the slow metabolism characterizing cells and tissue in hypothermic conditions is the reason for the limited HO-1 induction. However, the combination of curcumin and hemin was more effective than the two inducers alone, suggesting that: 1) different intracellular mechanisms are utilized by hemin and curcumin to induce HO-1 and, 2) relatively high levels of endogenous HO-1 can be achieved when using the right experimental approach, even in cells exposed to hypothermic conditions. In addition, the potential cytoprotective action of HO-1 may not necessarily require very high levels of the protein and may already be adequate when HO-1 expression is slightly above control values. It should be noted that hypothermic cells were treated with curcumin and/or hemin in Celsior solution rather than normal medium, to mimic more closely the cold storage conditions applied to organs for transplantation. Therefore, it cannot be excluded that Celsior solution could interfere with the ability of curcumin or hemin to induce HO-1.

Curcumin markedly reduced the damage caused by hydrogen peroxide to cardiac cells incubated at normal temperature and inhibition of heme oxygenase activity abrogated this effect. These data indicate the importance of the interaction between curcumin and the HO-1 pathway in protection against oxidative stress in physiological conditions and confirm results that we have obtained previously in other cell types (Motterlini *et al.,* 2000b; Scapagnini *et al.,* 2002; Balogun *et al.,* 2003b). The cold storage protocol utilized to allow HO-1 induction by curcumin or hemin did not cause injury in terms of cell viability or LDH release, but significantly increased the cell death as measured by the trypan blue exclusion method. The presence of curcumin considerably decreased cell damage, suggesting that curcumin retains its protective action at low temperature. To further investigate this possibility, cells were subjected to a longer cold storage period (6 h) in Celsior solution and also to cold storage followed by re-warming in medium for 1 h, a protocol which reproduces the reperfusion phase. Curcumin decreased markedly the cold storage and re-warming-induced injury, sustaining the idea that this natural compound could be used as a protective agent in cold storage solutions. Most strikingly, curcumin nearly completely prevented the extensive damage caused by prolonged (up to 24 h, data not shown) cold storage. This result is of special significance because it suggests that curcumin could be potentially used to prolong the cold storage time without compromising tissue viability.

It has to be noted that the different assays used in the present work to determine cell damage did not always produce agreeable results. This can be explained by the different nature of the assays. Specifically, the Alamar blue assay assesses cell viability by measuring the metabolic activity of the cells under culturing conditions, while the LDH release assay is an index of damage to the cellular membrane resulting in LDH leakage. Thus, although certain stimuli or experimental conditions might affect cellular metabolism and this type of signal could be detected by the Alamar blue assay, the same stimuli or conditions might be ineffective on cellular membranes and LDH release would remain unchanged. An opposite scenario might also occur with stimuli that cause an increase in LDH release without affecting cellular metabolism. Similar considerations can be made for Alamar blue or LDH release methods when compared to the trypan blue assay. It is expected that the best agreement on the output of the three assays can be obtained in conditions of extreme cytotoxicity when cellular metabolism, membrane integrity and cell viability would be considerably altered from normal conditions. In the case of our study, the three assays were in good accord, with the Alamar blue and LDH release assays show-

ing a high level of agreement.

One of the questions we have tried to address in this work is the contribution of HO-1 and heme oxygenase activity to curcumin-mediated cardiac cells protection. HO-1 is a very effective defensive system against oxidative stress-induced cardiac cell damage (Foresti *et al.,* 2001) as well as cardiac ischemia-reperfusion injury and organ rejection (Soares *et al.,* 1998; Clark *et al.,* 2003; Motterlini *et al.,* 2005) In addition, both CO and bilirubin, the two major products of heme degradation by heme oxygenase, have been demonstrated to exert a direct and significant protective effect both in cardiac tissue (Clark *et al.,* 2000b, 2003) and during cold preservation of organs (Sandouka *et al.,* 2006). Therefore, HO-1 induction was expected to play a major role also in the experimental protocol that we have employed in the present study. However, although we have shown that under normothermic conditions HO-1 is indeed strongly involved in the beneficial effects exerted by curcumin, we observed only a small contribution of HO-1 in hypothermia. This effect was confirmed using both an inhibitor of heme oxygenase activity and siRNA for HO-1; therefore, there are clearly other features intrinsic to the curcumin molecule that are responsible for its protective action under hypothermic conditions and should be further investigated. Interestingly, supplementation of patients with high doses of bioflavonoid capsules (480 mg curcumin and 20 mg quercetin) for 1 month after kidney transplant resulted in an improvement of early graft function and decreased acute rejection in a recently published randomized placebo controlled trial (Shoskes *et al.,* 2005). The authors also found that induction of HO-1 in cells collected from urine was correlated to the intake of bioflavonoids. Together with our previous data (Balogun *et al.,* 2003a), the present results indicate that there might be various applications for curcumin in the field of transplantation, from the early times of organ storage to the actual treatment of patients following transplantation procedures.

In conclusion, this study sustains an important beneficial role of curcumin added to Celsior preservation solution in the defense of human cardiac cells against the damage induced by cold storage and re-warming. HO-1 contributes to some extent to this protection and it is likely that the antioxidant properties of curcumin elicit a major defensive effect at low temperature. It is now necessary to establish the feasibility of using curcumin in preservation solutions in organ and animal investigations.

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