A BRIEF COMMUNICATION

Copper Inhibition of Hydrogen Peroxide–Induced Hypertrophy in Embryonic Rat Cardiac H9c2 Cells

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Previous studies have shown that dietary copper deficiency causes cardiac hypertrophy and depression of vascular epithelial growth factor (VEGF) expression in mouse model. Copper replenishment in the diet reverses cardiac hypertrophy and restores VEGF expression. The present study was undertaken to specifically determine the role of VEGF in copper effect on cell hypertrophy. Embryonic rat cardiac H9c2 cells were exposed to hydrogen peroxide to develop hypertrophy, determined by increases in cell size and total protein content. Copper addition at 5 μ*M* in cultures suppressed cell hypertrophy. In the presence of anti-VEGF antibody, copper inhibitory effect on cell hypertrophy was blunted, and VEGF alone mimicked the inhibitory effect of copper. The results thus demonstrated that VEGF is critically involved in copper inhibition of cell hypertrophy induced by hydrogen peroxide in the H9c2 cells. Exp Biol Med 232:385–389, 2007

Key words: copper; hypertrophy; VEGF; protein synthesis; H9c2 cells

Introduction

Copper, an important trace element for humans and animals, was first established for its nutritional essentiality in 1928 (1). It has been known that the concentration of copper is crucial for cardiac morphology and function (2–4). Dietary copper deficiency in animal models results in myocyte hypertrophy, electrocardiograph alteration, increased collagen accumulation, and lipid deposition in the myocardium (5–8). The hypertrophic cardiomyopathy induced by dietary copper deficiency can further develop to heart failure (6). Furthermore, cardiac tissue is sensitive to marginal dietary copper restriction, and even in the absence of any systemic signs of copper deficiency cardiac morphological and functional defects can occur (9–11). An interesting observation is that copper replenishment in the diet reverses hypertrophic cardiomyopathy induced by copper deficiency in mouse model (12).

How does copper exert its role in the regression of hypertrophic cardiomyopathy? It has been shown that copper is involved in vascular endothelial growth factor (VEGF) expression in cultured human keratinocytes (13). The gene expression of VEGF is significantly depressed in the hypertrophied myocardium induced by dietary copper deficiency in mice, and this depression is recovered after copper replenishment in the diet (14). Interestingly, both copper deficiency and VEGF deletion in mice cause cardiomyopathy, including impaired contractile function, mitochondrial structural and functional damage, and blunted myocardial responses to β-adrenergic stimulation (6, 9, 15).

It is thus interesting to determine the role of VEGF in copper manipulation of cardiac hypertrophy. In the present study, an embryonic rat cardiac H9c2 cell line was used to study the effect of copper on cell hypertrophy induced by hydrogen peroxide. In this oxidant-induced cell hypertrophy model, copper supplementation to the culture media at physiologically relevant levels inhibited the increase in cell size and suppressed the elevation of protein levels in the cell. Anti-VEGF antibody blunted the inhibitory effect of

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copper, and VEGF mimicked the copper effect. It thus suggests that copper inhibition of hydrogen peroxide–induced cell hypertrophy is mediated at least in part by stimulation of VEGF signaling pathway.

Materials and Methods

Cell culture. Embryonic rat cardiac H9c2 cells (purchased from the American Type Culture Collection, CRL-1446, Manassas, VA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis., MO) supplemented with 10% fetal bovine serum (FBS; Atlanta Biologicals, Lawrenceville, GA), 100 U/ml penicillin, and 100 μg/ml streptomycin. Cells were seeded at a density of 80,000 cells per 35-mm culture dishes for experimental procedures. All cell cultures were kept at 37°C in humidified air with 5% CO_2.

Experimental Model and Procedure. Cell hypertrophy was induced by hydrogen peroxide following the procedure previously described (16) with some modifications. Briefly, after culturing for 24 hrs, cells were treated with H_2O_2 at a final concentration of 100 μ*M* in culture medium. The level of H_2O_2 was determined from our preliminary study testing the range from 40–150 μ*M*, and at the selected level H_2O_2 caused a maximal hypertrophic effect with little cell death. After 2-hr incubation, H_2O_2 and the oxidized medium were removed from the cells by changing the medium. The cells were then incubated for 18 hrs, and cellular hypertrophy was observed both morphologically and biochemically, as described below.

Copper (at a final concentration of 5 μ*M* in the medium and in the form of $CuSO_4$) was added at the end of 18-hr incubation after 2-hr H_2O_2 exposure and present in the medium for 48 hrs. Then cells were counted using a hemocytometer after collection via trypsinization and suspension in phosphate buffered saline (PBS) buffer. Cells were collected and suspended in a lysis buffer (20 m*M* Tris-HCl, pH 7.5; 150 m*M* NaCl; 1 m*M* Na_2EDTA; 1 m*M* EGTA; 1% Triton; 2.5 m*M* sodium pyrophosphate; 1 m*M* beta-glycerophosphate; 1 m*M* Na_3VO_4; 1 μg/ml leupeptin with freshly added 1 m*M* phenylmethylsulfonylfluoride). To assess the role of VEGF in copper inhibition of cell hypertrophy, we used monoclonal anti-VEGF antibody (R & D Systems, Inc., Minneapolis, MN) at a concentration of 2 ng/ml, which was preincubated 1 hr before administration of copper. The effect of VEGF was further studied in the presence of 200 ng/ml recombinant rat $VEGF_{164}$ (R & D Systems), substituting for copper in the medium.

Protein Assay. Protein concentrations were measured by Bradford method using a commercially available assay kit according to the manufacturer's instruction (Bio-Rad, Hercules, CA) and a Beckman DU 650 spectrophotometer (Pegasus Scientific, Frederick, MD). Protein content per cell was determined by dividing the total amount of protein by the cell number and was expressed as micrograms per 10^6 cells.

Assessment of Morphological Changes. Medium was removed from the adherent H9c2 cell cultures followed by washing with PBS. Cells were fixed in 2.5% formalin in PBS for 10 mins, and then stained with hematoxylin and eosin (Sigma Chemical Co.). Morphological changes were visualized by phase contrast microscope. Images were acquired by Nikon digital camera DXM 1200 (Nikon Instruments, Melville, NY) using Nikon ACT-1 software.

Determination of Cell Volume. Cell volume was determined in detached cells obtained by trypsinization, as described previously (16). Briefly, rounded cells were loaded onto a microslide field finder (Fisher Scientific, Pittsburgh, PA). The diameters of 100–120 cells from each group were assessed and recorded. The cell volume was calculated using the equation for the volume of a sphere: $V = (4/3)\,\pi r^3$, where V = cell volume, $\pi = 3.14$, and r = radius.

Statistical Analysis. Data are expressed as mean ± SD, and each experiment was repeated three times with consistent results. Student's t test was used to compare the difference between two groups (Fig .1). A 2×2 factorial design was applied to the experiments presented in Figure 2 (copper vs. anti-VEGF antibody) and Figure 3 (H_2O_2 vs. VEGF), and the data were analyzed accordingly. After a significant interaction was detected by the two-way analysis of variance (ANOVA), the significance of the main effects against control was further determined. The level of significance was considered when $P < 0.05$.

Results

Cell Hypertrophy Induced by H_2O_2. After exposure to 100 μ*M* H_2O_2 in cultures, the H9c2 cells remained intact and showed no morphological changes at the end of the pulse treatment (data not shown). After incubation for 18 hrs following the H_2O_2 exposure, a significant enlargement of the cell size was visualized (Fig. 1). The nucleus of H9c2 cells underwent polyploidy and division, consistent with the observation reported previously (17). The average volume measured from the diameter of H9c2 cells was increased more than 2-fold in the H_2O_2-treated relative to untreated controls (Fig. 1). The number of H_2O_2-treated cells in cultures was not decreased after the treatment for 2 hrs and incubation for additional 18 hrs (data not shown). To evaluate whether or not the increase in cell volume was caused by elevated organic cellular components or merely cell swelling, protein assay was performed, and the total protein content was normalized relative to the total cell numbers. The normalized protein content in H_2O_2-treated cells was significantly increased; up to 2-fold compared with that of control, indicating an increase in protein synthesis (Fig. 1).

Inhibition by Copper of the H_2O_2-Induced Cell Hypertrophy. Incubation of the H_2O_2-treated H9c2 cells for additional 48 hrs resulted in further increases in cell volume and protein content relative to the cells treated with

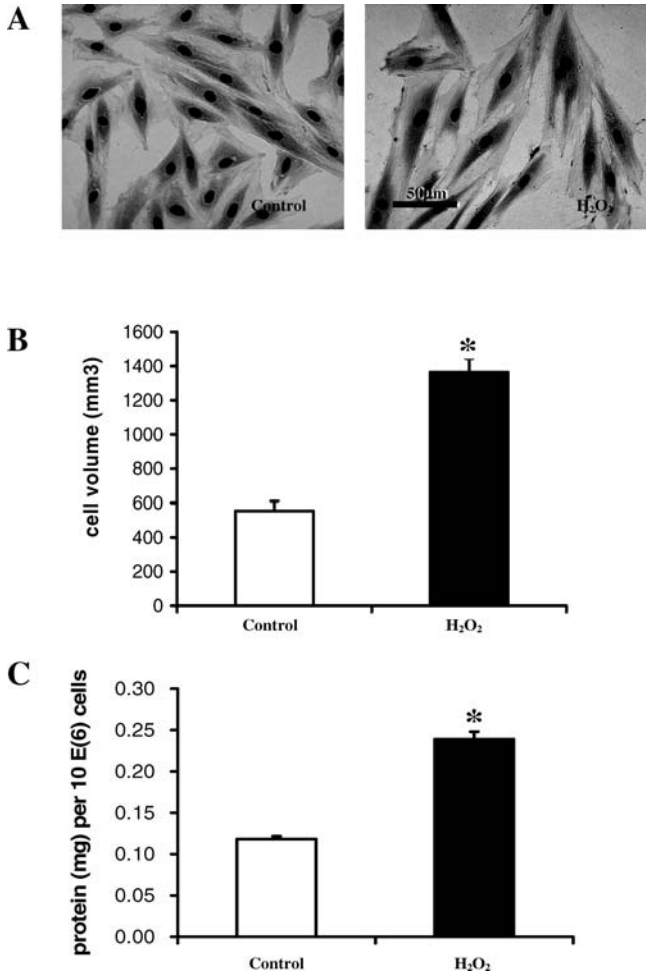

Figure 1. H_2O_2-induced cell hypertrophy in embryonic rat cardiac H9c2 cells. H9c2 cells in DMEM containing 10% FBS were treated with H_2O_2 at a final concentration of 100 μM in cultures for 2 hrs, and then the cells were incubated with fresh medium for 18 hrs. (A) Morphological observation of cell size increase in response to H_2O_2 treatment. (B) Cell volume changes measured after trypsinization and resuspension of the cells harvested after treatment with H_2O_2 for 2 hrs, then incubated for additional 18 hrs. (C) Protein content in the cells measured after the cells were treated with H_2O_2 for 2 hrs and incubated for an additional 18 hrs. $*$, significantly different from control group.

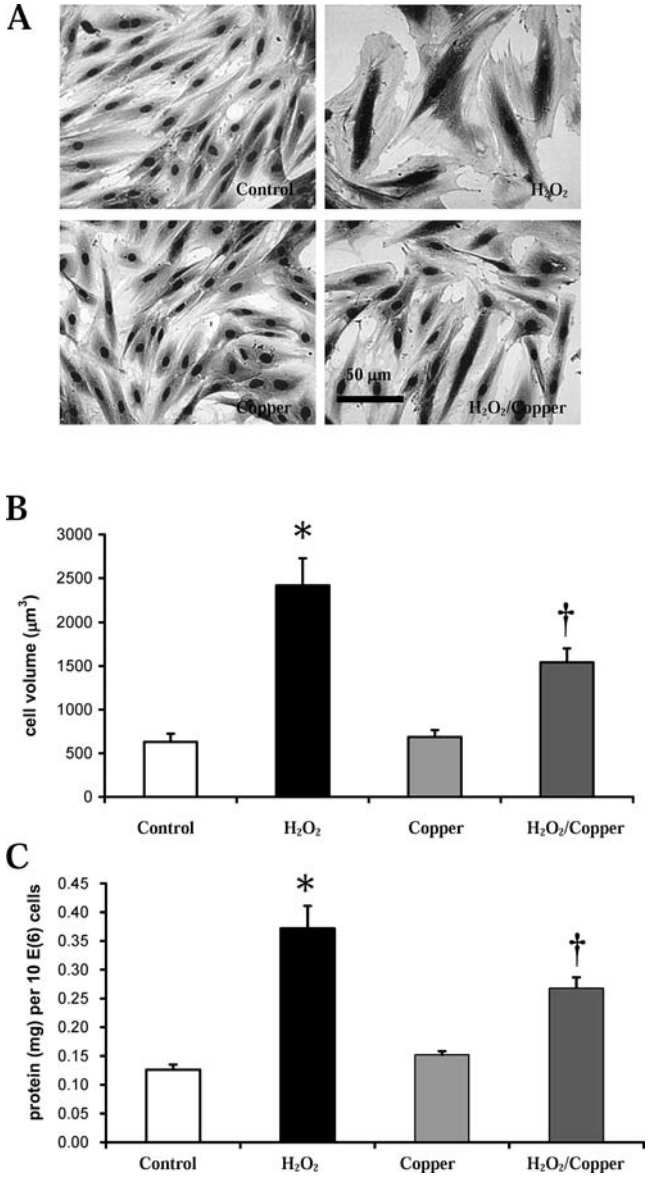

Figure 2. Copper inhibition of cell hypertrophy induced by H_2O_2. H9c2 cells in DMEM containing 10% FBS were treated with H_2O_2 at a final concentration of 100 μM in cultures for 2 hrs, and then the cells were incubated with fresh medium for 18 hrs. At the end of incubation, copper at a final concentration of 5 μM in the cultures was added, and the cells were further incubated for 48 hrs. (A) Morphological observation of cell size changes in response to H_2O_2 treatment and copper supplementation. (B) Cell volume changes measured at the end of 48-hr incubation with copper in the medium. (C) Protein content in the cell measured at the end of 48-hr incubation with copper. $*$ or †, significantly different from control group; †, significantly different from H_2O_2-treated group.

H_2O_2 and incubated for 18 hrs. In the presence of 5 μM copper in the cultures, the cell size and protein content were not further increased but remained comparable to that of cells treated with H_2O_2 for 2 hrs and incubated for 18 hrs (Fig. 2). Copper treatment alone did not cause significant changes in cell size.

VEGF Requirement for Copper Effect on Hydrogen Peroxide–Induced Cell Hypertrophy. Because copper is involved in the regulation of VEGF production (13), the possibility that copper inhibition of hydrogen peroxide–induced cell hypertrophy may be mediated by VEGF signaling system was examined. First, we used anti-VEGF antibody at the same time when the cultures were added copper (18 hrs after the cells were exposed to H_2O_2), then determined the effect on cellular protein content. Anti-VEGF antibody at 2 ng/ml in cultures did not change the effect of H_2O_2-induced cell hypertrophy, nor affect the protein content in untreated control cells, but blunted the inhibitory effect of copper on cell hypertrophy (Fig. 3). To further evaluate the involvement of VEGF in inhibition of cell hypertrophy, we used VEGF at 200 ng/ml to replace copper in the medium and found that VEGF indeed

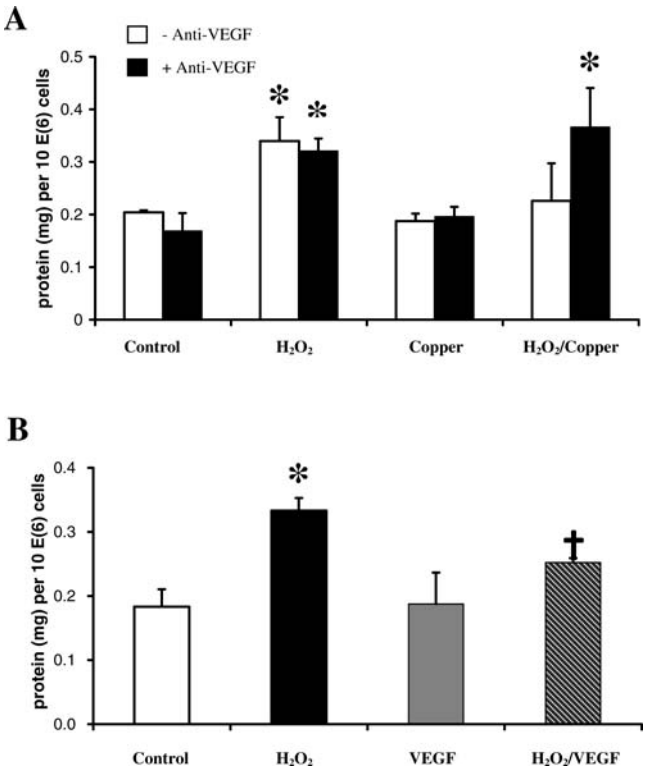

Figure 3. (A) Effect of anti-VEGF antibody on copper inhibition of cell hypertrophy induced by H_2O_2. H9c2 cells in DMEM containing 10% FBS were treated with H_2O_2 at a final concentration of 100 μM in cultures for 2 hrs, and then the cells were incubated with fresh medium for 18 hrs. At the end of incubation, copper at a final concentration of 5 μM in the cultures was added. Anti-VEGF antibody at a final concentration of 2 ng/ml was added 1 hr before copper addition. The cells were then incubated for additional 48 hrs before harvesting. (B) Effect of VEGF on H_2O_2-induced cell hypertrophy. H9c2 cells in DMEM containing 10% FBS were treated with H_2O_2 at a final concentration of 100 μM in cultures for 2 hrs, and then the cells were incubated with fresh medium for 18 hrs. At the end of incubation, recombinant rat $VEGF_{164}$ at a final concentration of 200 ng/ml was added to the cultures, and the cells were further incubated for additional 48 hrs. $*$ or †, significantly different from control group; †, significantly different from H_2O_2-treated group.

mimicked the effect of the copper inhibition of cell hypertrophy (Fig. 3).

Discussion

The results obtained here demonstrated that copper inhibition of hypertrophy in the embryonic rat cardiac H9c2 cells recaptures the *in vivo* observation that copper replenishment in the diet reverses hypertrophic cardiomyopathy. However, there is fundamental difference between the *in vivo* and *in vitro* models. Cardiac hypertrophy was induced by dietary copper restriction *in vivo*, while cell hypertrophy was induced by hydrogen peroxide *in vitro*. The trigger for dietary copper deficient hypertrophy is the effect of the lack of copper on copper–dependent enzymes and proteins, while the trigger for hydrogen peroxide–induced hypertrophy is oxidation. However, it has been shown in both cases that hypertrophic signaling transduction pathways and the fetal gene program are activated (18, 19). Copper exerts inhibitory effect on hypertrophy induced by both copper deficiency (12) and oxidants (present study). Therefore, it is important to identify the crucial signaling pathway that leads to the inhibitory effect. The important finding from this study is that VEGF is critically involved in copper inhibition of cell hypertrophy induced by hydrogen peroxide.

Previous studies have shown that dietary copper restriction suppresses cardiac VEGF expression in association with heart hypertrophy and copper replenishment in the diet restores VEGF expression along with the reversal of hypertrophic cardiomyopathy (12, 14). It has been known that copper stimulates VEGF expression in cells (13, 20), but the important question is whether or not VEGF mediates the effect of copper. In the present study, we have specifically addressed this question by using anti-VEGF antibody to block the effect of VEGF in copper-treated hypertrophic cells. Since VEGF action is mediated by VEGF receptor through autocrine or paracrine mechanisms, anti-VEGF antibody efficiently blocks the VEGF receptor mediated signaling pathway (21). The essential role of VEGF signaling pathway in copper inhibition of cell hypertrophy induced by hydrogen peroxide was demonstrated by the blockade effect of the anti-VEGF antibody. Furthermore, when VEGF was used in the culture medium to replace copper, the same inhibitory effect on cell hypertrophy was observed. Therefore, the results from both the anti-VEGF antibody and the VEGF alone demonstrate the involvement of VEGF signaling pathway in copper inhibition of cell hypertrophy by hydrogen peroxide.

It is important to note that the level of copper used in the present study is physiologically relevant. The addition of 5 μM copper to the culture medium would be comparable to serum level of copper in humans provided with recommended dietary allowance (RDA) for copper at 0.9 mg/day. Unfortunately, marginal copper restriction in humans, defined as consumption of a diet resulting in less than the RDA level, has been identified from surveys of food consumption (22–24). The detrimental effect of marginal copper deficiency has been demonstrated in a recent study that shows in the absence of commonly observed signs of copper deficiency, the heart suffers from both morphological and functional injury from marginal dietary copper deficiency in adult rats (9–11). Although dietary copper deficiency causes heart hypertrophy and the transition to heart failure (6), replenishment of adequate levels of copper in diet is sufficient to reverse the hypertrophic cardiomyopathy and prevent heart failure (12). This is further confirmed by the *in vitro* results presented here that addition of physiologically relevant levels of copper in cultures inhibits cell hypertrophy induced by hydrogen peroxide.

Given that VEGF plays a critical role in copper inhibition of cell hypertrophy, the next important question is how VEGF mediates the effect of copper. Recent studies have shown the importance of VEGF in prevention of

cardiac pathologic hypertrophy in a mouse model with sustained pressure overload (25–27). Sustained pressure overload causes heart hypertrophy and contractile dysfunction, which is correlated with a disruption of coordinated angiogenesis (25, 26). In addition, exogenous supplementation of VEGF prevents or reverses cardiomyopathy in several mouse models of cardiac ischemic or pressure overload diseases (28, 29). In copper deficient heart hypertrophy, mitochondrial proliferation contributes to the enlarged cardiomyocytes (2). It remains to further investigate the molecular mechanisms, including the effect of VEGF on mitochondria in the reversal of hypertrophic cardiomyopathy.

In summary, the present study demonstrates that copper at physiologically relevant levels inhibits cell hypertrophy induced by hydrogen peroxide in embryonic rat cardiac H9c2 cells. The copper inhibitory effect is mediated at least in part by VEGF signaling pathway because anti-VEGF antibody blunts and VEGF alone mimics the copper inhibition of cell hypertrophy. Further studies are required to understand molecular mechanisms by which VEGF inhibits heart hypertrophy.

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