Copper supplementation restores cytochrome *c* oxidase activity in cultured cells from patients with *SCO2* mutations

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Human SCO2 is a nuclear-encoded Cu-binding protein, presumed to be responsible for the insertion of Cu into the mitochondrial cytochrome *c* oxidase (COX) holoenzyme. Mutations in *SCO2* are associated with cardioencephalomyopathy and COX deficiency. Studies in yeast and bacteria have shown that Cu supplementation can restore COX activity in cells harbouring mutations in genes involving Cu transport. Therefore we investigated whether Cu supplementation could restore COX activity in cultured cells from patients with *SCO2* mutations. Our data demonstrate that the COX deficiency observed in fibroblasts, myoblasts and myotubes from patients with *SCO2* mutations can be restored to almost normal levels by the addition of $CuCl_2$ to the growth medium.

Key words: assembly, cardioencephalomyopathy, COX deficiency, mitochondria, respiratory chain.

INTRODUCTION

Cytochrome *c* oxidase (COX), or complex IV of the mitochondrial respiratory chain, comprises 13 subunits, two haem groups (*a* and a_3), three Cu ions (two in the Cu_A site and one in the Cu_B site), a Zn ion and an Mg ion. The three larger subunits (COX I–III), encoded by mitochondrial DNA, comprise the catalytic core of the enzyme and contain the prosthetic groups. The ten smaller subunits are encoded by nuclear DNA and have a regulatory and structural function [1].

The assembly of COX requires a number of ancillary proteins that control the correct folding and maturation of the various polypeptides, as well as the delivery and insertion of the prosthetic groups into the holoenzyme. Several yeast COX assembly genes and their human homologues have been identified. Mutations in four of these genes – *SCO1* [2], *SCO2* [3], *COX10* [4] and *SURF1* [5] – have been associated with COX deficiency in patients, resulting in a variety of clinical phenotypes. *COX10* is required for COX haem biosynthesis, but the function of *SURF1* is presently unknown.

SCO1 and SCO2 are involved in the insertion of Cu atoms into the Cu_A site on the COX II subunit [6,7]. Previous studies [8–10] of the analogous yeast genes suggest that Cu is shuttled by COX17p, a metallochaperone, from the cell membrane to the mitochondrial intermembrane space, where it releases Cu to SCO1p or SCO2p, which then assemble it into the COX II polypeptide. The exact relationship between SCO1 and SCO2 is still under investigation, but it is quite probable that both bind Cu. In particular, both proteins have a highly conserved CXXXC motif similar to the CXXXC motif present at the Cu_A site of COX II, suggesting an analogous Cu-binding function for both SCO1 and SCO2 [3]. A third protein, COX11, is probably required for Cu delivery to the Cu_B site on COX I [11].

We observed mutations in the *SCO2* gene in a group of patients with a fatal form of cardioencephalomyopathy and COX deficiency [3]. These patients exhibit a very severe hypertrophic cardiomyopathy that is usually fatal within the first year of life, an encephalopathy similar to Leigh syndrome, and myopathy. COX activity in skeletal muscle, heart and brain is less than 10% of controls, but the defect is less severe in other tissues, such as liver or fibroblasts, where it is approx. 50% of controls. No treatment is currently available for this disorder. However, several studies have shown that Cu supplementation can bypass defects in genes of the Cu delivery pathway. Studies in *Bacillus subtilis* have shown that strains that are mutant in *ypmQ*, a gene similar to the human *SCO* genes, are deficient in COX activity, but that normal COX levels can be restored by growth in Cu-supplemented media [6]. In yeast, COX deficiency associated with mutations in *COX17* can be restored by high Cu concentrations in the culture [8], and a similar effect was shown in yeast strains with mutations in CCS, a Cu chaperone that is required for the assembly of Cu–Zn-superoxide dismutase [12]. Therefore we investigated whether Cu supplementation in the growth medium of cultured cells from patients with *SCO2* mutations could restore COX activity.

MATERIALS AND METHODS

Patients

Skin biopsies were obtained from normal controls, from a patient (P1) with heterozygous mutations in *SCO2* (Q53X/E140K) (patient no. 2 in ref. [3]) and from a patient (P2) with heterozygous mutations in *SURF1* (828delCTex9/855delCTex9) (patient no. 5 in ref. [13]). Muscle biopsies were obtained from a patient (P3) with heterozygous mutations in *SCO2* (E140K/dup1302–1311) [14].

Cell culture

Fibroblasts were grown from explant cultures of skin biopsies as monolayer cultures in complete Minimum Essential Medium (Invitrogen, Carlsbad, CA, U.S.A.) supplemented with 15% foetal bovine serum (FBS), 100 mg/ml streptomycin and 100 units/ml penicillin. Myoblasts were obtained by trypsin dissociation of muscle biopsies followed by selective plating to

Abbreviations used: COX, cytochrome *c* oxidase; CS, citrate synthase; FBS, foetal bovine serum.

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eliminate fibroblasts, and were grown in the same growth medium. Coverslips were placed in the cell culture dishes in order to perform biochemical, histochemical and immunocytochemical assays in parallel.

Since myoblasts from patient P1 were not available, fibroblasts from controls and patients P1 and P2 were converted into myoblasts by retroviral transfection of the muscle differentiation gene, *MyoD*, as described earlier [15]. These myoblasts were maintained in culture in the same growth medium as described above.

In order to obtain a pure population of myoblasts from primary muscle cultures from patient P3, fibroblasts were removed by successive complement fixation, using antibody 1B10 to the surface protein of fibroblasts (a gift from Dr Barton Haynes, Duke University Medical Center, NC, U.S.A.) as described earlier [16]. This procedure resulted in cultures containing more than 85 % myoblasts.

Differentiation of confluent myoblasts was induced by growing for 4–6 weeks in mitogen-depleted medium, DMEM F-14, supplemented with BSA (0.5 mg/ml) and insulin, transferrin, selenium supplement (Invitrogen, New York, NY, U.S.A.). Cultures of fibroblasts, myoblasts or myotubes, 80–90 % confluent, were trypsinized and pelleted for biochemical assays.

Cu supplementation

$CuCl_2$ was preferred to $CuSO_4$ because the sulphate precipitated in cell culture medium which contains 15 % FBS. $CuCl_2$ was added to the complete culture medium before the addition of FBS, to avoid precipitation. To determine the optimum concentration of Cu required to restore COX activity, 4×10^5 cells were plated in several 100-mm² dishes and incubated for 24 h before adding growth medium supplemented with 0, 50, 100, 150 and 200 μM $CuCl_2$ for 10 days. On day 11, the cells were trypsinized and pelleted for enzyme assays. To determine the optimum time for Cu supplementation, 4×10^5 cells were seeded in six 100-mm² dishes, and 200 μM $CuCl_2$ was added to the medium in each dish for 0, 2, 4, 6, 8 and 10 days, respectively. Cells were harvested on day 11 and biochemical assays were performed. To determine the specificity of the effect of Cu, biochemical quantification of COX was performed on fibroblasts from a normal subject and from patient P1 after 10-day supplementation with 0, 100 and 200 μM of either $ZnCl_2$ or $CuCl_2$.

Biochemistry

Cell pellets were resuspended in PBS and lysed by two freeze–thaw cycles. COX activity was measured by the method of Capaldi and co-workers [17] with slight modifications. Bovine cytochrome *c* was reduced with ascorbate and then desalted using a Sephadex G-25 column (Amersham). The concentration of the reduced substrate was determined spectrophotometrically at a wavelength of 565 nm. Citrate synthase (CS) activity was measured by the method of Srère [18]. All spectrophotometrical measurements were performed using a Cary UV 100 spectrophotometer (Varian Inc., Walnut Creek, CA, U.S.A.).

During the course of this work, we observed that Cu added at levels of 50 μM or greater inhibited the activity of CS, a mitochondrial-matrix enzyme often used for normalizing respiratory-chain activities in cells, by approx. 60 % in both patient and normal cell lines. Therefore we did not use CS activity to normalize COX activities, but normalized to protein content of the lysates. The values were reported relative to COX activities determined in control cells treated concurrently in an identical manner. Statistical significance was calculated using Student's *t* test.

Histochemistry and immunocytochemistry

In histochemical staining for COX activity, the cells grown on glass coverslips in the same dishes used for enzyme activity measurements were air-dried for 1 h at room temperature, preincubated with 1 mM $CoCl_2$ and 50 μl of DMSO in 50 mM Tris/HCl, pH 7.6, containing 10 % sucrose, for 15 min at room temperature. After rinsing with PBS, the cells were incubated for 6 h at 37 °C with 10 ml of substrate solution (10 mg of cytochrome *c*, 10 mg of diaminobenzidine, 2 mg of catalase and 25 μl of DMSO in 10 ml of 0.1 M phosphate buffer, pH 7.6). The coverslips were rinsed in PBS, mounted in glycerin–gelatin and were examined with a Zeiss microscope with bright-field optics.

For double immunolabelling, the cells were fixed with 4 % paraformaldehyde in PBS, pH 7.4, containing 0.1 % (v/v) Triton X-100 for 30 min at room temperature, incubated for 1 h with a rabbit polyclonal antibody to subunit II of human COX (a gift from Dr Russell Doolittle, University of California, San Diego, CA, U.S.A.), rinsed with PBS, and then incubated with a Texas-Red-labelled secondary antibody (Molecular Probes Inc., Eugene, OR, U.S.A.) for 1 h. The coverslips were then rinsed with PBS before adding a monoclonal antibody to COX subunit IV (a gift from Dr Armand Miranda, Columbia University, New York, NY, U.S.A.), incubated for 1 h, rinsed with PBS and then incubated with the secondary antibody conjugated with FITC (Molecular Probes). For studies of muscle differentiation, myotubes cultured on glass coverslips in mitogen-depleted medium were immunostained with a monoclonal antibody to sarcomeric myosin heavy chain (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, U.S.A.), followed by FITC-labelled anti-mouse secondary antibody (Molecular Probes Inc.). On serial-muscle-biopsy sections from control and patient P3, immunohistochemical analysis was performed as described earlier [19]. Fluorescence was visualized with a Zeiss fluorescence microscope with epi-illumination optics and the images were captured with appropriate filters using the NIH image analysis software (Scion Corporation).

RESULTS

Concentration of $CuCl_2$ and COX activity

We studied the effect of different $CuCl_2$ concentrations on cell lines from a normal control and from those with mutations in *SCO2* and *SURF1* (Table 1). Biochemical analysis showed a clear dose-dependent effect of Cu supplementation on COX activity in both fibroblasts (Figure 1A) and *MyoD*-transformed fibroblasts (Figure 1B) from patient P1 with *SCO2* mutations, whereas we found no effect of the Cu supplementation on COX activity in the fibroblasts and *MyoD*-transformed fibroblasts from a control and *SURF1* patient P2. The initial COX activity in the *SCO2* fibroblasts was 55 % of the value in normal controls in untreated cells, and was virtually unaltered by treatment with 50 μM $CuCl_2$. However, higher concentrations of $CuCl_2$ improved COX activity, up to 92 % ($P < 0.001$) at 200 μM $CuCl_2$. Levels higher than 200 μM produced no further increase in COX activity. Similarly, COX activity was decreased in myoblasts (50 % of controls) and myotubes (44 % of controls) from patient P3. Supplementation with 200 μM $CuCl_2$ increased COX activity to 99 % of control values in myoblasts ($P < 0.001$) and to 82 % ($P < 0.001$) in myotubes. The measured activity was virtually 100 % cyanide-sensitive (results not shown), ruling out a possible non-specific oxidant effect of Cu on the substrate, reduced cytochrome *c*. Fibroblasts from a control and from patient P1 were also treated with 0, 100 and 200 μM $ZnCl_2$

Table 1 COX activity after $CuCl_2$ treatment

The Table shows COX activity after Cu supplementation in normal control, *SCO2* patient P1, *SURF1* patient P2 and *SCO2* patient P3. n.s., non-significant.

		Activity [nmol · min^{-1} · (mg of protein)$^{-1}$] after $CuCl_2$ (μM) supplementation					*t* test, *P* value after $CuCl_2$ (μM) supplementation		
Cell line	*n*	0	50	100	150	200	0 versus 100	0 versus 200	100 versus 200
Fibroblasts									
Control	4	77 ± 2	77 ± 3	79 ± 2	82 ± 5	78 ± 2	n.s.	n.s.	n.s.
Patient P1	4	42 ± 2	45 ± 2	53 ± 5	61 ± 2	70 ± 3	< 0.005	< 0.001	< 0.005
Patient P2	4	7 ± 1	7 ± 2	7 ± 2	8 ± 1	5 ± 2	n.s.	n.s.	n.s.
MyoD-transformed fibroblasts									
Control	3	63 ± 2		60 ± 4		62 ± 2	n.s.	n.s.	n.s.
Patient P1	3	34 ± 2		44 ± 3		57 ± 2	< 0.02	< 0.001	< 0.001
Patient P2	3	9 ± 2		9 ± 2		9 ± 2	n.s.	n.s.	n.s.
Myoblasts									
Control	3	69 ± 2		72 ± 4		75 ± 5	n.s.	n.s.	n.s.
Patient P3	3	34 ± 3		54 ± 4		68 ± 3	< 0.005	< 0.001	< 0.01
Myotubes									
Control	3	91 ± 3		86 ± 4		88 ± 4	n.s.	n.s.	n.s.
Patient P3	3	40 ± 3		59 ± 3		75 ± 4	< 0.005	< 0.001	< 0.01

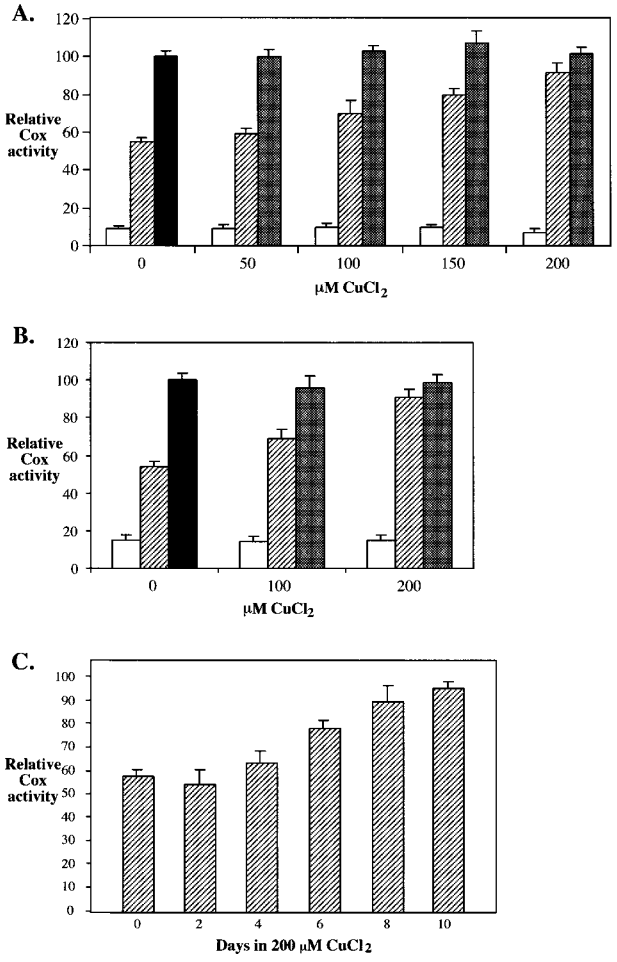

Figure 1 COX activity after Cu supplementation in cells from *SCO2* patient P1 (hatched shading) or *SURF1* patient P2 (no shading) compared with control cells (grey shading)

All COX activities are normalized to the value in control cells with no added Cu (black shading). Fibroblasts (**A**) and *MyoD*-converted myoblasts (**B**) were grown for 10 days in a medium containing Cu. (**C**) COX activity in myoblasts from patient P3 after treatment with 200 μM $CuCl_2$ for 0–10 days.

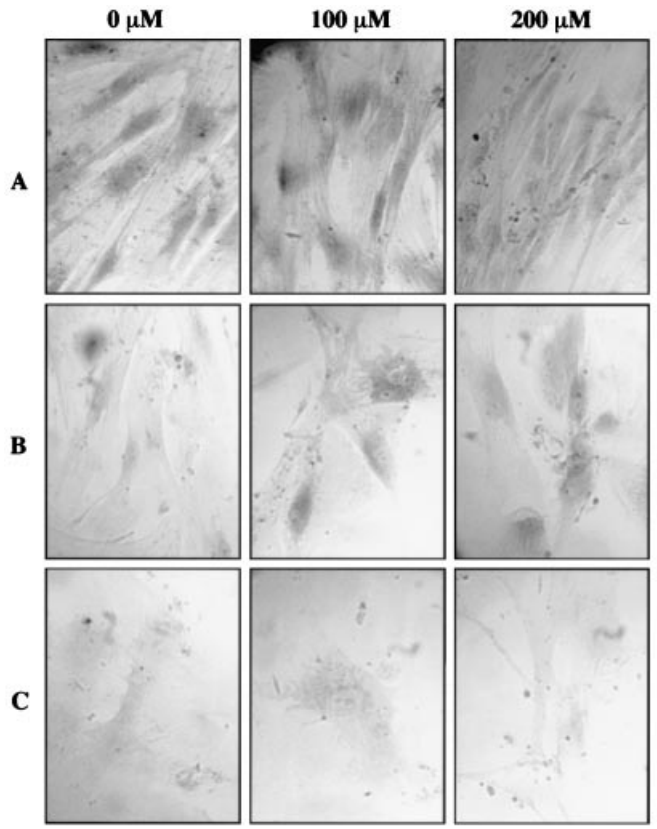

Figure 2 COX histochemistry of *MyoD*-converted fibroblasts, after treatment with 0, 100 and 200 μM $CuCl_2$

(**A**) Control, (**B**) *SCO2* patient P1 and (**C**) *SURF1* patient P2.

for 10 days, as above. In contrast with the Cu treatment, the addition of Zn to the medium was unable to elicit an increase in COX activity, thus demonstrating that the effect was apparently specific to Cu (results not shown).

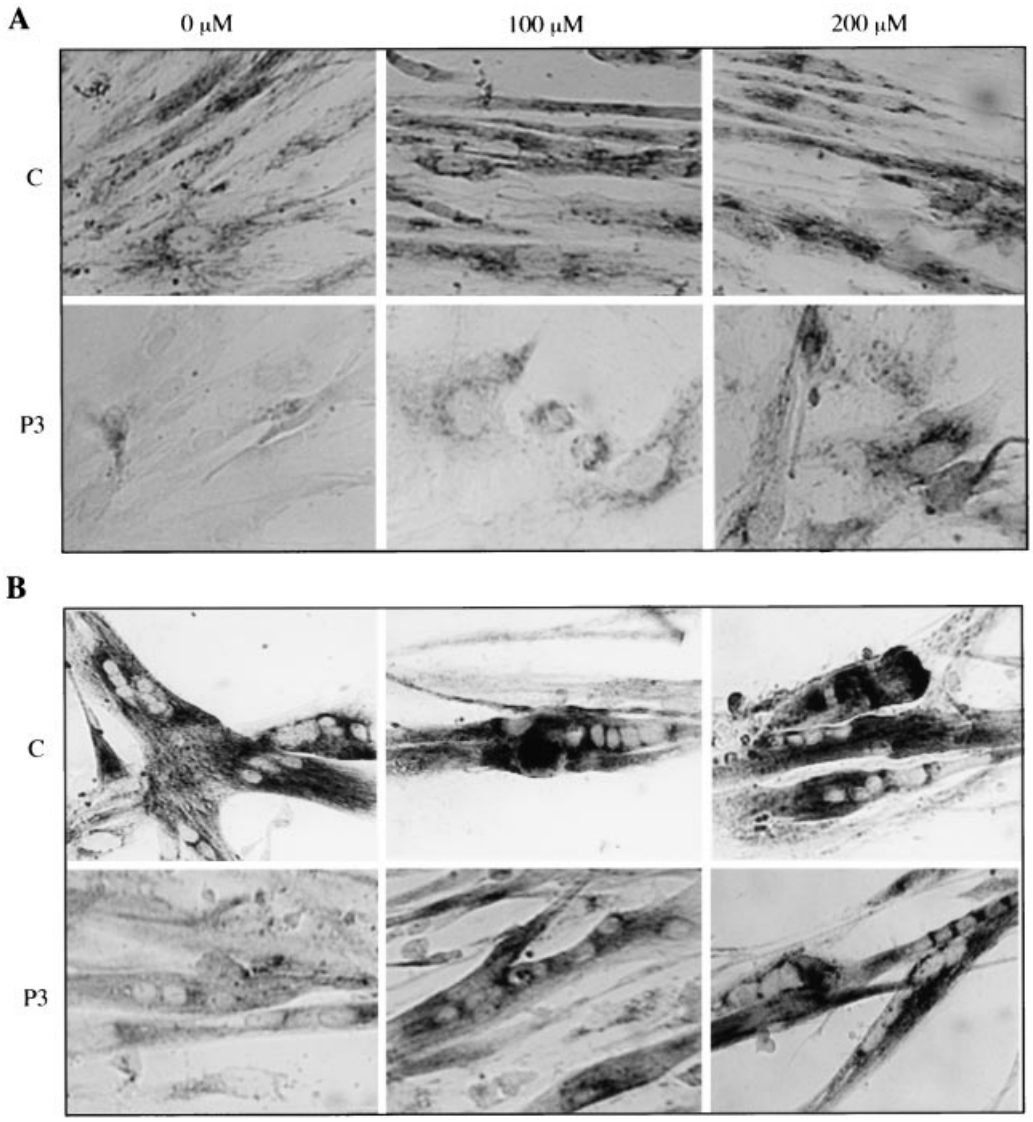

Figure 3 COX histochemistry of myoblasts (A) and myotubes (B) from SCO2 patient P3 after treatment with 0, 100 and 200 μM $CuCl_2$ (C is control)

To investigate the amount of time necessary for the Cu treatment to result in maximum recovery of COX activity, the cells were incubated in complete medium supplemented with 200 μM $CuCl_2$ for different time periods. The increase in COX was initially slow, gradually reaching maximum levels (95% of controls) after 8–10 days (Figure 1C). Longer incubation periods resulted in no further increase in COX activity.

Histochemical analysis

The dose-dependent increase in COX activity that we observed by biochemical assay was also observed with histochemical staining for COX activity of cultures grown in parallel with those cultured for biochemistry. As with the biochemical data, Cu supplementation had little or no effect on COX in normal fibroblasts or in fibroblasts from the patient with *SURF1* mutation (results not shown) as well as in the corresponding *MyoD*-transformed cell lines (Figure 2). However, there was a dose-dependent increase in the intensity of COX histochemical staining pattern with added Cu in both fibroblasts (results not shown), as well as in the fibroblasts transformed with *MyoD* (Figure 2) from *SCO2* patient P1.

Primary myoblasts cultured from a muscle biopsy obtained from a second patient with *SCO2* mutations (patient P3) also demonstrated a low-intensity-staining pattern for COX histochemistry as compared with control myoblasts, and a concomitant increase in the intensity of staining following Cu supplementation (Figure 3A). When switched to mitogen-depleted medium, the myoblasts from P3 fused to form multinucleated myotubes at the same rate and efficiency as myoblasts from a normal control. The expression of sarcomeric myosin heavy chain, a marker of muscle differentiation, was also normal by immunocytochemistry (results not shown). Histochemical staining of myotubes revealed COX deficiency, which was restored almost to control levels by Cu supplementation (Figure 3B). Although COX activity in cells supplemented with 200 μM

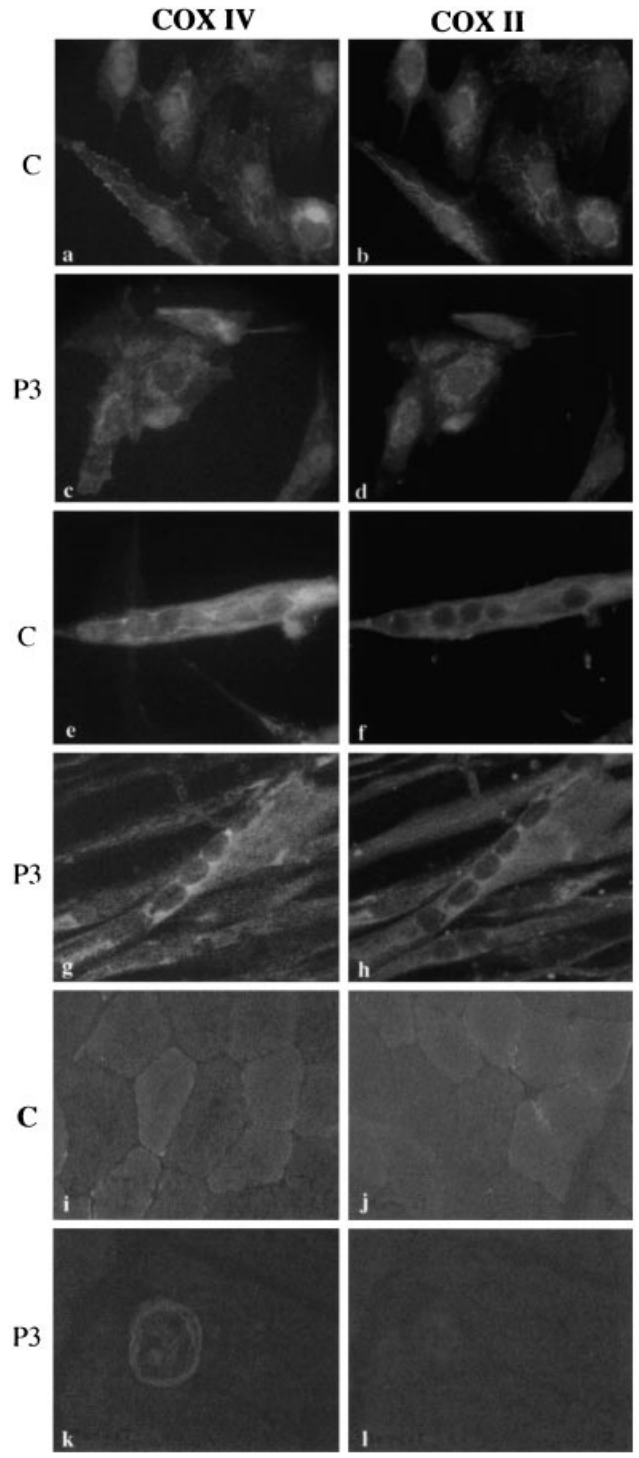

Figure 4 Immunocytochemistry of myoblasts (a–d), myotubes (e–h) and muscle sections (i–l) from control (C) and from *SCO2* patient P3 (P) using a mouse monoclonal antibody to COX IV subunit and a rabbit polyclonal antibody to COX II subunit

Cu was significantly greater than that in cells supplemented with 100 μM Cu, this difference was not seen by histochemistry, probably because of the lower sensitivity of the latter technique, which was not designed to be quantitative.

Immunocytochemistry

Both myoblasts and myotubes from the *SCO2* patient P3 revealed normal steady-state levels of the mtDNA-encoded COX II subunit and the nuclear DNA-encoded COX IV subunit, by immunocytochemical analysis, whereas muscle sections from patient P3 showed significantly reduced staining of both subunits compared with control (Figure 4). With COX IV antibody, the muscle capsule showed some positive staining in Patient P3. Cu supplementation of myoblasts and myotubes did not show any further change in the immunostaining patterns (not shown).

DISCUSSION

Cu is essential for the activity of several key enzymes in cells, but at the same time it is a potentially dangerous compound because it can catalyse a number of redox reactions that produce toxic free radicals. Cells therefore closely regulate the traffic of Cu, maintaining the precise amount necessary for biological function to avoid toxicity, and in fact there is essentially no free Cu in the cell [12]. The handling of Cu is carried out by a class of proteins called Cu chaperones, which mediate the delivery of the ion from the plasma membrane to subcellular compartments, where it is needed for the correct assembly of several enzymes [20]. This process has been well characterized for Cu–Zn-superoxide dismutase, which requires the Cu chaperone CCS for its assembly [21].

COX17 is required for Cu delivery to the mitochondrial inner membrane, where it delivers Cu to a second group of Cu chaperones, the SCO proteins and possibly COX11, which insert the metal into the nascent COX polypeptides. Mutations in *SCO1* and *SCO2* in humans, and in *COX17* in yeast, have been shown to cause COX deficiency, probably because they disrupt the normal Cu delivery pathway. COX deficiency due to mutations in *COX17* in yeast [8], and mutations in *ypmQ* [6] (the *SCO* homologue in *B. subtilis*), can be rescued by increasing the Cu levels in the culture medium.

These data prompted us to supplement Cu in the growth medium of cells obtained from *SCO2* patients with COX deficiency. We observed a restoration of COX activity in these cultured cells with addition of $CuCl_2$ to the growth medium. This effect was apparently specific for cells with *SCO2* mutations, as we observed no increase in COX activity in cells from patients with *SURF1* mutations, which are severely COX-deficient due to an impairment in the COX assembly pathway, presumably unrelated to Cu metabolism [22].

The range of Cu concentration used in our studies was not toxic to the cells. Cu concentrations range from 10.5 to 23 μM in human serum [23] and from 20 to 25 μM in cell culture medium supplemented with 15% FBS [24]. Cu toxicity varies widely among different cell types. Human dermal fibroblasts do not show signs of toxicity when incubated in the presence of Cu concentrations up to 500 μM [24]. However, we did not exceed the concentration of 200 μM $CuCl_2$, in order to minimize the possible toxic effects on cells in culture. Treatment with $CuCl_2$ at concentrations ranging from 50 to 200 μM for 10 days produced no variation in COX activity in a normal cell line. However, CS, a nuclear-encoded protein, was reduced to 60% of the levels in untreated cells at all Cu concentrations tested. Cu is known to strongly inhibit CS as well as other enzymes of the tricarboxylic acid cycle such as aconitase and NAD– and NADP–isocitrate dehydrogenases in *Aspergillus niger* [25]. Citrate is a potent organic chelator of metals, like Cu; therefore Cu may reduce 'free citrate', resulting in apparently lower CS activity. *In vitro*, Cu appears to exert an inhibitory effect on both NAD- and FAD-dependent enzymes, such as succinate dehydrogenase and

phosphofructokinase [26,27]. Therefore, COX activities could not be normalized either with CS or with succinate dehydrogenase, which is routinely done.

The mechanism by which Cu stimulated COX activity in SCO2-deficient cells is unknown. We note, however, that we did not have a true *SCO2* null cell line. In fact, both patients were compound-heterozygous for a mutation (either nonsense or frameshift) encoding truncated proteins that lack the putative Cu-binding domain on one allele, and a missense *E140K* mutation on the other allele. This latter mutation probably still encodes a partially functional SCO2 polypeptide [28], as supported by the fact that the analogous mutation in yeast does not cause a COX-deficient phenotype [7], whereas Cu treatment could not rescue an *SCO1* null mutant in yeast [9]. Moreover, a patient who was homozygous for the *E140K* mutation had a relatively milder (albeit ultimately fatal) course compared with patients with the E140K mutation on only one allele [29]. Thus it is likely that the observed effect of Cu is mediated by this partially functional protein (i.e. E140K), in which the substitution of a negatively charged glutamate residue with a positively charged lysine adjacent to the putative CXXXC Cu-binding motif reduces (but does not abolish) the affinity of the protein for Cu cations. Alternatively, the mutation might impair either the interaction between SCO2 and COX17 or between SCO2 and the COX II Cu_A site.

We note that we exposed the cells to Cu concentrations ten times higher than the physiological ones. Such an overload probably saturated the Cu-buffering capacity of intracellular chaperones, leading to an increased number of free Cu ions in the cell from virtually zero to measurable amounts [12]. Probably, several mechanisms may be operative in this Cu-overloaded environment. First, the increased free Cu may compensate for the reduced affinity of SCO2 for Cu. Secondly, increased Cu may upregulate synthesis of COX17 or other Cu chaperones (including SCO2 itself) and therefore the delivery of Cu to SCO2 or to COX II. Data in yeast, however, do not support the notion that COX17 can interact directly with COX II [7]. Finally, as alluded to above, the availability of increased free Cu could allow the direct insertion of Cu into the Cu_A site.

Our cell culture model is not ideal because it cannot reproduce the full COX-deficient phenotype seen in patient's muscle *in vivo*. Myotubes still retained 44 % control COX activity in contrast to the 5 % or less measured in muscle biopsies of the same patient [14]. Furthermore, whereas muscle biopsies from *SCO2* patients exhibit a selective decrease in COX I and COX II subunits by immunocytochemistry [3], in our studies with muscle cultures we were unable to demonstrate a decrease in COX II by immunocytochemistry, either in myoblasts or in myotubes (Figure 4) probably because cultured myotubes are not differentiated to the same degree as muscle *in vivo*. Nevertheless, COX deficiency of myoblasts from patients with *SCO2* mutations did not seem to impair the fusion of myoblasts to form myotubes. Differentiation seemed to occur at rates similar to control myoblasts, as indicated by the normal level of expression of sarcomeric myosin heavy chain by immunocytochemistry (results not shown). It is interesting to note that fibroblasts from patient P1 had ≈ 50 % COX activity similar to that of *MyoD*-transformed fibroblasts from patient P1 and myoblasts from patient P3, and demonstrated a similar response to Cu that was similar to what we observed in *MyoD*-transformed fibroblasts from patient P1 and myoblasts from patient P3. In spite of these similarities, patients with *SCO2* mutations do not exhibit any overt clinical phenotype in fibroblasts or liver. It is therefore apparent that there may be some unknown mechanism by which the *SCO2* mutations affect only certain tissues. One obvious possibility is that the expression of *SCO1* can somehow compensate for the defects in *SCO2* in a tissue-specific manner. This possibility is currently being investigated.

Interestingly, the restoration of normal COX activity by Cu is a gradual process that required several days to become evident. There was an initial lag phase, after which there was a slow increase in activity, reaching 95 % of control levels after 10 days, in an apparently linear fashion (Figure 1C). This type of behaviour implies that SCO2 does not behave catalytically (e.g. one polypeptide of SCO2 inserts multiple atoms of Cu into COX in a cyclical manner), but rather behaves as a Cu-binding and transport protein in which one polypeptide of SCO2 delivers one atom of Cu to COX, which may then be degraded. Cells synthesize COX holoproteins continuously, but in SCO2-deficient cells only a fraction of these holoproteins are assembled correctly due to a dysfunction in Cu delivery. Thus in SCO2-deficient cells, there is an equilibrium between the synthesis of COX II polypeptides and the degradation of the subset of COX II polypeptides that do not assemble properly. Cu is somehow able to shift this equilibrium, favouring the assembly and thus the accumulation of the correctly folded subunits.

Many questions remain on the relationship between *SCO1* and *SCO2*. Both genes seem to be expressed ubiquitously at the mRNA level [3], but no data are available yet on the pattern of protein expression. Both *SCO1* and *SCO2* mutations cause severe COX deficiency in muscle [2,3], but apparently only *SCO1* is essential in liver, whereas COX deficiency in the liver of a *SCO2* patient is relatively mild and is comparable with that in fibroblasts. Unfortunately, there are no data available on COX activity in fibroblasts of *SCO1* patients.

The fact that Cu can rescue COX activity in patient cells immediately raises the issue of the possible therapeutical applications of this observation. However, one must bear in mind that our results have been obtained in a cell culture model and that we have exposed cells to Cu concentrations that would be lethal for a human being. Further studies in animal models of *SCO2* mutations will be helpful to explain the mechanism of this effect and to determine whether lower Cu concentrations could still be beneficial for patients. These experiments are currently in progress.

Note added in proof (received 18 February 2002)

Since the present paper was submitted, a paper by Jaksch et al. [30] has appeared. In the study reported, they supplemented Cu in the culture medium of myoblasts from a patient with *SCO2* mutations and found restoration of COX deficiency to normal levels.

We thank Dr E. Bonilla for help with muscle immunohistochemistry and Dr M. Hirano for helpful discussions. This work was supported by grants from the U.S. National Institutes of Health [NS28828 and NS39854 (E.A.S.) and HD32062 (M.M.D. and E.A.S.)] and Telethon Italia Grant no. 439b (L.S.).

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Received 18 October 2001/6 December 2001; accepted 19 January 2002