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Copper Downregulates Neprilysin Activity Through Modulation of Neprilysin Degradation

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ABSTRACT

Copper plays a central role in conserved processes such as respiration, and in highly specialized processes, such as protein modification. The metalloprotease neprilysin (NEP) degrades a variety of bioactive peptides and is involved in many physiological processes. However, very little is known about the regulation of NEP activity. In the current study, we focused on the effect of Cu^{2+} on the enzymatic activity and protein stability of NEP. Using mouse neuroblastoma N2a cells, we found that the enzymatic activity of NEP was decreased by treatment with Cu^{2+} in a dose- and time-dependent manner. In our investigation of the mechanism by which Cu^{2+} downregulates NEP enzyme activity, we found that treatment with Cu^{2+} caused a decrease in the level of NEP as determined by Western blotting analysis. Quantitative analysis of NEP mRNA with RT-PCR excluded the possibility that Cu^{2+} downregulates NEP protein at the gene transcription level. Moreover, specific proteasome inhibitors, MG132 and lactacystin, blocked the turnover of NEP, whereas inhibitors of lysosome had no significant effect, suggesting that Cu^{2+} induced degradation of NEP is via a proteasome pathway. Taken together, our data suggest that copper downregulates NEP activity through modulation of NEP protein degradation.

Keywords: activity, copper, degradation, neprilysin

INTRODUCTION

Most Alzheimer's disease (AD) cases occur randomly in the population, and many risk factors might be involved in the development of AD. A number of metal ions have been found to be risk factors associated with the pathogenesis of AD. For example, aluminum in drinking water has been reported to increase the risk of AD [1], and the neuronal toxicity of amyloid-β (Aβ) is partly mediated by interaction of the peptide with iron [2]. Among these biometals, the most important and complicated one is copper. There is considerable evidence that the homeostasis of copper and several metalloproteinases is disturbed in AD [3, 4]. Serum and cerebrospinal fluid (CSF) copper levels have been reported to be elevated in AD patients [5, 6], while the activities of some copper-dependent enzymes show the opposite trend to copper level. For examples, cytochrome C-oxidase and peptidylglycine α-amidating monooxygenase show decreased activities in AD patients [3, 7]. Copper can bind to histidine residues of Aβ, accelerate the aggregation of Aβ, and subsequently mediate the neurotoxicity of the peptide [8-12].

Copper is an essential transition biometal and is a co-factor for a number of metalloenzymes. Neprilysin (NEP, EC 3.4.24.11) is a metalloprotease which is involved in many physiological processes, such as neuropeptide metabolism, inflammation, cell migration, and proliferation [13, 14]. NEP has wide regulatory activities and degrades a variety of bioactive peptides, such as enkephalin and Aβ [14]. The involvement of NEP in Aβ degradation was first reported by Howell and colleagues [15]. Subsequently, using mouse models it was found that NEP is the major Aβ degrading enzyme in the brain [14, 16].

However, very little is known about the regulation of NEP activity, or the relationship between copper and NEP. In this study, we report that Cu^{2+} suppresses endogenous NEP activity in a dose-dependent manner and that the downregulation of NEP protein is mediated by the proteasome pathway.

MATERIALS AND METHODS

Reagents

Copper chloride ($CuCl_2$), thiorphan, Succinyl-Ala-Ala-Phe-7AMC, phosphoramidon, and microsomal leucine aminopeptidase were obtained from Sigma-Aldrich (St. Louis, MO, USA). The protease inhibitor cocktail "Complete, EDTA-free" was purchased from Roche Diagnostics (Mannheim, Germany) and MG132 from Calbiochem (San Diego, CA, USA). Wild type mouse neuroblastoma N2a cells and WT-7 cells (N2a cells stably expressing both Swedish mutant APP695 and human wild-type PS1) were kindly provided by Drs. Sangram S. Sisodia (University of Chicago) and maintained as described previously [17].

Copper treatment

Since Cu^{2+} has been commonly used at a concentration of 10 μM in similar studies [18], we first performed a time course experiment (0, 24, 48, and 72 h) using 10 μM Cu^{2+}. A dose curve experiment was performed by incubating cells with different concentrations of Cu^{2+} (0, 2.5, 10, and 30 μM) at 37°C for 48 h.

NEP enzymatic activity assay

Cells were plated in 6 well-plates. After treatment with Cu^{2+}, the medium was removed, and cells were rinsed with PBS buffer. 200 µl of 2-(N-morpholino) ethanesulfonic acid (MES) buffer containing 1% Triton X-100 was then added, and cells were incubated for 15 min on ice as described previously [19, 20]. Cells were then scraped and homogenized by passing them through a 20-gauge needle 30 times. For each experiment, 30 µg of protein were incubated with or without Thiorphan, a neprilysin inhibitor, in black-colored 96-well plates (Corning) with the protease inhibitor cocktail Complete, 10 µM MG132 and 0.1 mM Succinyl-Ala-Ala-Phe-7AMC (substrate of NEP) in DMSO. After incubating for 60 min at 37°C, 10 µM phosphoramidon and 5 µg/ml microsomal leucine aminopeptidase were added. After a second incubation for 30 min at 37°C, fluorescence absorbance was recorded using a FLUOStar fluorometer (BMG Lab Technologie, Germany) at an emission wavelength of 460 nm and an excitation wavelength of 390 nm.

Western blot analysis

Western blot analysis was carried out as previously described [19-21] with slight modification. First, cells were harvested on ice, homogenized, and suspended in RIPA buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, and Complete protease inhibitor cocktail) for analysis of NEP. For analysis of amyloid-β protein

precursor (AβPP) and AβPP- C-terminal fragments (CTF), homogenized cells were suspended in PARP buffer (50 mM Tris-HCl, pH 6.8, 8 M urea, 5% β-mercaptoethanol, 2% SDS, and protease inhibitors). The protein concentration of each homogenate was determined using a BCA kit (Pierce). Samples (120 μg protein/well) were separated by 10% SDS-PAGE or 10% Bicine/urea SDS-PAGE. After being transferred to a polyvinylidene fluoride membrane (Millipore), NEP, β-actin and AβPP were probed with primary antibodies, namely NEP-specific monoclonal antibody (1:1,000 dilution, R&D, Minneapolis, MN), β-actin-specific monoclonal antibody (1:10000 dilution, Sigma) and AβPP-specific monoclonal antibody 6E10 (1:1000 dilution, COVANCE, Dedham, MA) with overnight incubation at 4°C. Membranes were then washed in TBS (containing 0.075% Tween 20) and incubated with HRP-conjugated goat anti-mouse IgG secondary antibody (Jakson). Blots were developed with an ECL detection system (Supersignal Pico Western system, Pierce).

RT-PCR analysis

Total RNA was extracted with TRIZOL (Invitrogen) and converted to cDNA by reverse transcriptase using random hexamers to prime superscript III RNasefree reverse transcriptase (Invitrogen) according to the manufacturer's instructions. Each RT-PCR primer used in this study was as follows; neprilysin sense primer, 5'-AGCCTCTCTGTGCTTGTCTTGC-3'; neprilysin antisense primer, 5'-CACTCATAGTAGCCT-CTGGAAGGG-3'; β-actin sense primer, 5'-TGTACGCCTCTGGCCGTACC-3'; and β-actin antisense primer,

5’-CCACGTCACACTTCATGATGG-3’. PCR reactions were performed at 94°C for 30 s, 55°C for 1 min, and 68°C for 2 min during 40 cycles, followed by a final extension of 7 min at 68°C [20]. The resulting PCR products were analyzed on a 1% agarose gel stained with ethidium bromide.

Aβ quantification

The levels of $A\beta_{40}$ in conditioned medium were measured with an ELISA kit (BIOSOURCE Immunoassay Kit), according to the manufacturer’s instructions. Medium samples were co-incubated with a rabbit antibody against $A\beta_{40}$ in the Kit plates with 3 h shaking. Media samples were then removed and secondary antibody was added. $A\beta_{40}$ was finally quantified by a color reaction using a plate reader (Bio-Rad, Model 680).

Statistical analysis

Data were calculated as a proportion of the control value and expressed as mean ± S.E. Comparisons between multiple groups were made by one-way ANOVA followed by Dunnett's Multiple Comparison Test, and the Student’s t test was used for comparisons between two groups (confidence limits of $p<0.05$). All statistical analyses were performed using Prism 4.0 software.

RESULTS

Copper downregulated neprilysin activity

In order to investigate whether Cu^{2+} is involved in regulating NEP activity, WT-7 cells were treated with 10 μM Cu^{2+} for different time periods. After 24 h, 48 h, and 72 h of treatment, cells were harvested and NEP activity was measured by fluorimetric assay. As shown in Fig. 1A, NEP activity was not changed after treatment of up to 24 h. However, at the 48 h time point, NEP activity decreased by 43.95%. At the 72 h time point, NEP activity showed a further slight additional decrease of 6.22%. Since treatment for 72 h did not result in any significant further decreases in NEP activity from that observed at 48 h, subsequent experiments were carried out by treating cells for 48 h.

We next evaluated the effects of different concentrations of Cu^{2+}on NEP activity. WT-7 cells were treated with 2.5, 10, and 30 μM of Cu^{2+} for 48 h. As shown in Fig. 1B, a Cu^{2+} dose dependent decrease in NEP activity was observed. Specifically, Cu^{2+} caused a decrease in NEP activity of over 40% even at the lowest concentration of 2.5 μM. When cells were treated with 30 μM of Cu^{2+}, NEP activity decreased to 28.97% of the basal level. To determine whether the decrease in NEP activity caused by Cu^{2+} is due to cell death, we examined the effect of Cu^{2+} on cell viability. Treatment of WT-7 cells with concentrations of Cu^{2+} of up to 30 μM showed no effect on the cell viability (data not shown). Therefore, these data clearly indicate that the decrease in NEP activity caused by treatment with Cu^{2+} was not due to cell death. We also treated wild type N2a cells with Cu^{2+} at different concentrations. As shown in Fig. 1C, Cu^{2+}

caused a similar dose dependent decrease in NEP activity, but to a lesser extent than that with WT-7 cells. In addition, when human neuroblastoma SH-SY5Y cells were used, the dose dependent decrease in NEP was the same as in N2a wild type cells (data not shown).

To determine the specificity of the Cu^{2+} induced decrease in NEP activity, we treated N2a cells with either Cu^{2+} alone, or with Cu^{2+} plus bathocuproine disulfonic acid (BCS), a Cu^{2+}-specific chelator. As shown in Fig. 1D, the addition of BCS blocked the Cu^{2+} induced decrease in NEP activity (compare third and second columns, Fig. 1D). To further determine the specificity of the Cu^{2+} induced decrease in NEP activity, we also treated the cells with magnesium chloride and we found that Mg^{2+} had no effect on NEP activity (Fig. 1E).

Copper caused a dose-dependent increase in secreted Aβ

Since NEP is one of the major Aβ-degradation enzymes previously reported [14], we next examined the effect of Cu^{2+} treatment on the level of Aβ. WT-7 cells were treated with 2.5 μM and 10 μM Cu^{2+} for 48 h. $A\beta_{40}$ secreted into the media was determined by ELISA. As shown in Fig. 1F, treatment with Cu^{2+} resulted in a dose-dependent increase in the levels of $A\beta_{40}$ in the media, and this finding is in agreement with the decrease in NEP activity. We also examined the effect of Cu^{2+} on the expression of AβPP and found that treatment with Cu^{2+} had no effect on AβPP processing (data not shown).

Copper caused a decrease in neprilysin protein level but had no effect on mRNA level

To investigate how Cu^{2+} modulates NEP activity, we determined whether Cu^{2+} had any effect on NEP expression, and we first examined the effect of Cu^{2+} treatment on the NEP protein level with Western blotting. N2a cells were treated with various concentrations of Cu^{2+} for 48 h, followed by Western blotting analysis. As shown in Fig. 2A and B, after treatment with Cu^{2+}, the NEP protein levels decreased in a dose dependent manner, dropping to 79.62% with 2.5 μM Cu^{2+}, 62.12% with 10 μM Cu^{2+}, and 49.80% with 30 μM Cu^{2+}. The β-actin level was unaffected by Cu^{2+} treatment. Thus, the β-actin also served as an internal protein loading control in this experiment. These results suggested that Cu^{2+}-induced decrease in NEP activity is due to downregulation of the NEP protein level.

To determine whether Cu^{2+} induced decrease in NEP protein level is due to downregulation of NEP gene expression, we next examined the effect of Cu^{2+} treatment on the level of NEP mRNA. N2a cells were treated with Cu^{2+} at different concentrations and NEP mRNA was analyzed by RT-PCR. As shown in Fig. 2C and Fig. 2D, treatment with Cu^{2+} showed no significant effect on the level of NEP mRNA, indicating that Cu^{2+} did not cause downregulation of NEP gene expression.

Cu^{2+} caused NEP protein degradation via the proteasome pathway

The data presented above suggested that the Cu^{2+}-induced decrease in the NEP protein level was not due to downregulation of NEP gene expression. This raised the possibility that Cu^{2+}

treatment results in an enhanced turnover or degradation of NEP protein.

To determine whether the Cu^{2+}-induced decrease in NEP activity was due to enhanced degradation of NEP by proteasomes, we examined the effects of the potent proteasome inhibitor MG132 and lactacystin on Cu^{2+}-induced decrease in NEP protein level. N2a cells were incubated in the presence or absence of MG132 or lactacystin for 1 h prior to the addition of $CuCl_2$. The NEP protein level was determined at 48 h following addition of 10 μM of Cu^{2+}. As shown in Fig. 3, cells treated without MG132 or lactacystin showed the expected reduction in NEP protein levels in response to Cu^{2+} (compare lane 2 with lane 1 of Fig. 3A). In contrast, in cells pretreated with MG132 or lactacystin, the NEP protein level remained largely unchanged upon treatment with Cu^{2+} (lanes 3 to 6, Fig. 3). However, the lysosomal protease inhibitor chloroquine did not block Cu^{2+}-induced NEP degradation. It is also observed that the NEP protein levels in MG132- and lactacystin-treated cells were higher than that in cells without treatment with these inhibitors. Taken together, these results indicate that the turnover or degradation of NEP protein is mediated by proteasome pathway. These data further strongly argue that the Cu^{2+}-induced decrease in NEP activity is a result of enhanced turnover or degradation of NEP protein, mediated by proteasomes.

DISCUSSION

It is recognized that copper plays a role in normal metabolism and in certain pathophysiologic conditions. Elevated extracellular copper has been demonstrated in the brain in

AD [4], however, the question as to whether and how the elevated Cu^{2+} contributes to the disease development remains elusive. In this study, we examined the effect of Cu^{2+} on the activity of NEP, which is believed to be one of the major Aβ degradation enzymes, and found that treatment with Cu^{2+} caused a marked decrease in NEP activity. The normal concentration of Cu^{2+} in CSF is from 0.01 to 5.7 μM [22], and in AD patients this level increased [5]. Therefore in our study, we used the concentrations of 2.5, 10, and 30 μM of Cu^{2+}, which are in the range of the concentrations of Cu^{2+} found under the disease conditions. It has been reported that at equilibrium, two to three copper ions (II) bind stoichiometrically to proteinase K (EC 3.4.21.14) and abolish its activity [23]. Our findings provide another example of copper directly affecting the activity of proteinase.

To address the question as to how Cu^{2+} causes a decrease in NEP activity, our data demonstrate that Cu^{2+} causes a significant decrease in the NEP protein level. Moreover, our data clearly indicate that the decrease in NEP protein level caused by Cu^{2+} treatment is not due to downregulation of NEP gene expression, but is instead due to enhanced turnover or degradation of NEP protein. There are two main pathways for physiologic protein degradation, namely the lysosome and proteasome pathways. In this study, by testing the proteasome-specific inhibitors MG132, and lactacystin and the lysosome inhibitor chloroquine, we demonstrated that Cu^{2+}-induced degradation of NEP was mediated by a proteasome pathway. Proteasome is one of the major intracellular devices in which damaged or unneeded proteins are broken down. Proteasome deals primarily with endogenous proteins that were synthesized within the cells but

folded incorrectly because of translation errors or other factors that cause posttranslational conformational changes of protein leading to its misfolding. There are two possibilities regarding the mechanism by which high dose of Cu^{2+} causes proteasomal degradation of NEP. One possibility is that Cu^{2+} directly binds to NEP and causes a conformational change on NEP and increases its susceptibility to proteasomal degradation; the other possibility is that treatment with Cu^{2+} results in an increase in proteasome activity, leading to accelerated turnover of NEP. However, at the present time, these possibilities are awaiting further examination in the future study. Recent studies have reported that copper posttranslationally regulates the levels of several proteins. Copper has been reported to stimulate the degradation of the copper chaperone of SOD1 protein, and this can be blocked by the proteasome inhibitors MG132 and lactacystin, but not by a cysteine protease inhibitor or inhibitors of the lysosomal degradation pathway [18]. The copper transporter protein hCtr1 is also posttranslationally regulated by copper. Elevated copper stimulates rapid endocytosis and degradation of hCtr1 [24]. Our study provides another example of a protein whose level is posttranslationally modulated by copper.

NEP is one of the most important enzymes involved in Aβ degradation. In this regard, it is particular interesting to note that treatment of cells with Cu^{2+} resulted in an increase in secreted Aβ. It is very likely that this increase in secreted Aβ upon Cu^{2+} treatment is a result of downregulation of NEP activity. Using a rabbit model it has been reported that copper introduced into the drinking water could induce Aβ plaque formation and learning deficits [25]. Moreover, several studies showed that elevation of serum free copper is correlated with cognitive decline in

AD [26, 27]. However, the mechanism by which copper regulates Aβ levels in such cases remains elusive. One possibility is that copper and zinc ions bind to Aβ and rapidly precipitate Aβ. Another possibility is that depletion of these biometals results in dissolution of Aβ deposits, and their degradation [10, 28-30]. The results which we report here raise another possibility, that copper may regulate Aβ levels by modulating the activity of the Aβ degradation enzyme NEP.

Many studies have reported the effect of Cu^{2+} on the pathogenesis of AD, however, the results are controversial. An increased extracellular copper has been reported in the brain of AD [4] and depletion of copper has been shown to downregulate expression of the AβPP gene [31]; whereas, other studies reported that copper inhibited Aβ production, possibly by altering the AβPP processing pathway [32] and that elevated intracellular level of copper caused reduction of Aβ likely due to upregulation of matrix metalloproteases in Chinese hamster ovary cells [33, 34]. The discrepancy of these observations may be due to different cell types respond differently to copper treatment.

In summary, we report for the first time that copper downregulates endogenous NEP activity. Moreover, our data demonstrate that copper suppresses NEP protein levels by modulating NEP degradation through a proteasome pathway. These results suggest a novel mechanism to explain how copper homeostasis affects the activity of metalloprotease and open a new avenue for studying the role of copper in amyloidosis in AD.

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FIGURE LEGENDS

Figure 1. Effects of Cu^{2+} on endogenous NEP activity and secreted $A\beta_{40}$. (a) WT7 cells were treated with $CuCl_2$ for 0, 24, 48, and 72 h, and NEP activity was evaluated as described in Materials and Methods.WT7 (b) and N2a (c) cells were incubated with different concentrations of $CuCl_2$ (2.5 μM, 10 μM, 30 μM), at 37ºC for 48 h, and NEP activity was evaluated as described in Materials and Methods. (d) N2a cells were grown in media alone, or in media with 10 μM $CuCl_2$, or 20 μM BCS for 48 h, and NEP activity was evaluated as described in Materials and Methods. Data are representative of three independent experiments. (e) Cells were treated with $CuCl_2$ or $MgCl_2$ for 48 h, and NEP activity was analyzed as described in Materials and Methods. (f) $A\beta_{40}$ levels in medium from Cu^{2+}-treated WT7 cells. Cultures were exposed to various concentrations of Cu^{2+} (0, 2.5, 10, 30 μM) for 48 h and $A\beta_{40}$ levels were measured in culture media by ELISA. Quantitative data are stated as mean ± SEM (n = 3); *$p < 0.05$ vs. control, **$p < 0.001$ vs. control.

Figure 2. NEP protein and mRNA levels under Cu^{2+} treatment. N2a cells were cultured with medium only or media with various concentrations of $CuCl_2$ (2.5 μM, 10 μM, 30 μM) for 48 h. (A) Cell lysates were immunoblotted for NEP (top panel) and β-actin was used as a loading control (bottom panel). (B) Protein levels were quantified and normalized to those of β-actin controls and expressed as percentages of control cell levels. Data are expressed as mean ± SEM

(n = 3). (C) RT-PCR of NEP mRNA. (D) NEP mRNA levels were quantitated and normalized to β-actin controls and expressed as percentages of control cell levels. Data are expressed as mean ± SEM (n = 3).

Figure 3. NEP protein after proteasome and lysosome inhibitors and Cu^{2+} treatment. (a) After 1 h of incubation with or without MG132 (1 μM), with or without lactacystin (4 μM), or with or without chloroquine (5 μM), $CuCl_2$ (0 μM, 10 μM) was added to the cells, and 48 h later, cells were examined for NEP expression by Western blotting (*top panel*). The membrane was probed for β-actin as a protein loading control (*bottom panel*). Data are expressed as mean ± SEM (n = 3), *$p < 0.05$ vs. control.

Figure 1

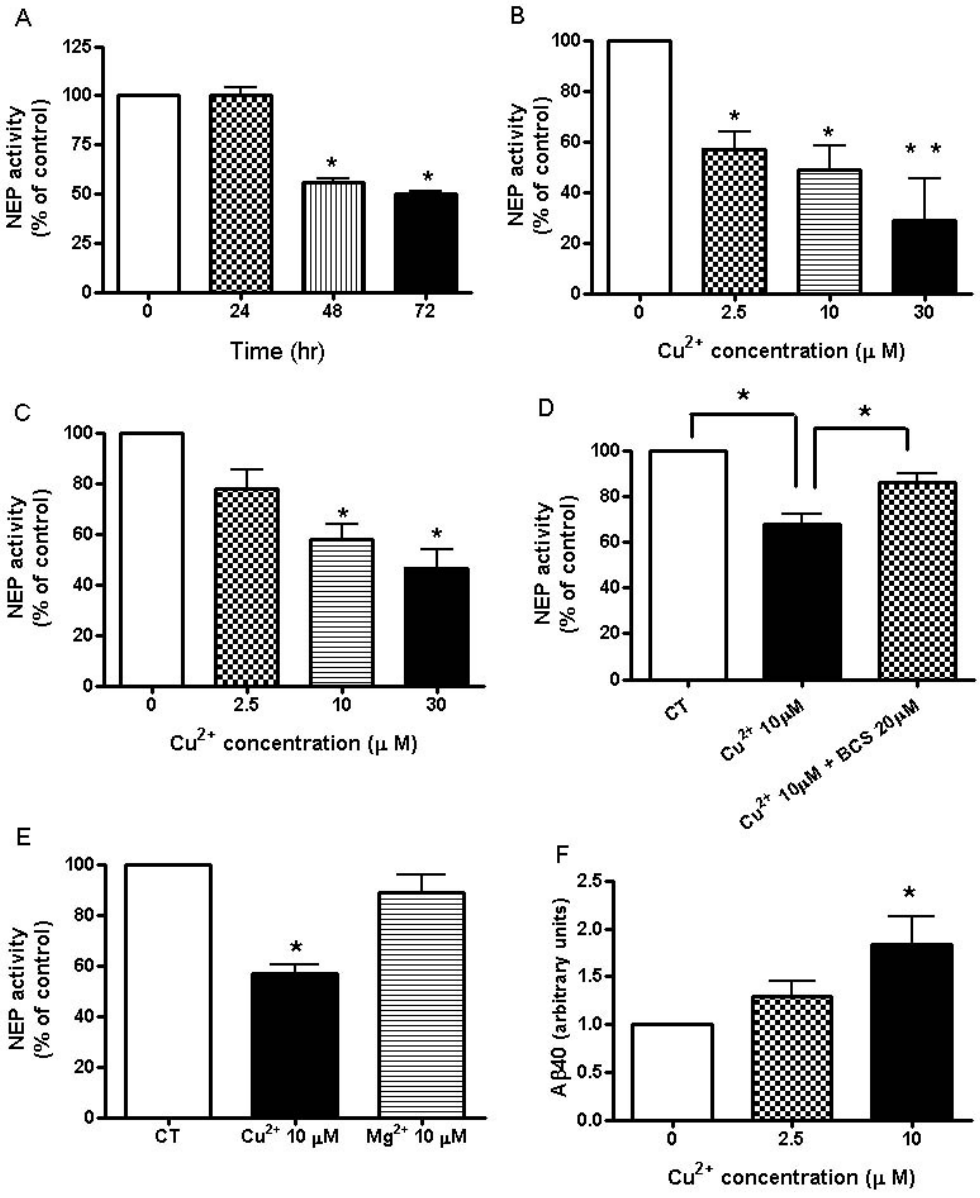

A
NEP activity (% of control)
125
100
75
50
25
0
0
24
48
72
Time (hr)
B
NEP activity (% of control)
100
80
60
40
20
0
0
2.5
10
30
Cu2+ concentration (μ M)
C
NEP activity (% of control)
100
80
60
40
20
0
0
2.5
10
30
Cu2+ concentration (μ M)
D
NEP activity (% of control)
100
80
60
40
20
0
CT
Cu2+ 10μM
Cu2+ 10μM + BCS 20μM
E
NEP activity (% of control)
100
80
60
40
20
0
CT
Cu2+ 10 μM
Mg2+ 10 μM
F
Aβ40 (arbitrary units)
2.5
2.0
1.5
1.0
0.5
0.0
0
2.5
10
Cu2+ concentration (μ M)

Figure 2

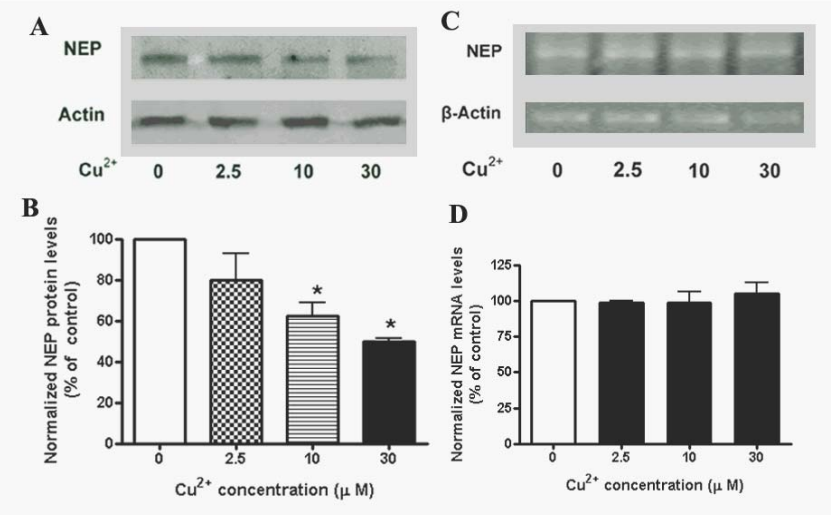
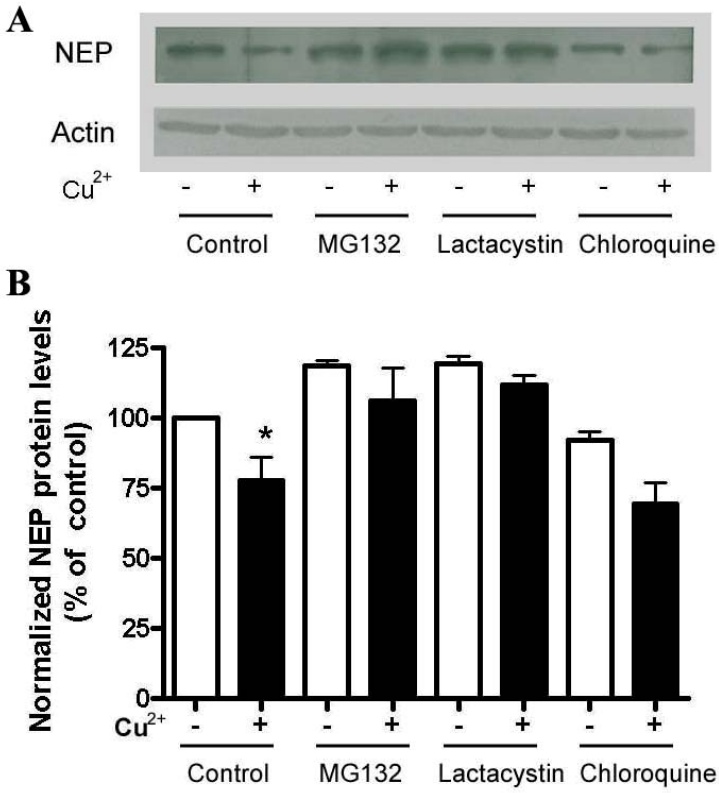

A
NEP
Actin
Cu2+ 0 2.5 10 30
C
NEP
β-Actin
Cu2+ 0 2.5 10 30
B
Normalized NEP protein levels (% of control)
100
80
60
40
20
0
*
*
0 2.5 10 30
Cu2+ concentration (μ M)
D
Normalized NEP mRNA levels (% of control)
125
100
75
50
25
0
0 2.5 10 30
Cu2+ concentration (μ M)

Figure 3


A
NEP
Actin
Cu2+
- + - + - + - +
Control
MG132
Lactacystin
Chloroquine
B
Normalized NEP protein levels
(% of control)
125
100
75
50
25
0
*
Cu2+
- + - + - + - +
Control
MG132
Lactacystin
Chloroquine