Changes in the expression of epigenetic factors during copper-induced apoptosis in PC12 cells

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Short title: Changes of epigenetic factors in apoptotic status

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Received November 16, 2013

ABSTRACT

Despite extensive research on copper toxicity the mechanisms involved are not fully characterized. There have been many recent reports concerning the relationship between epigenetic factors and cell metabolism, but the effects of copper exposure on epigenetic factor has not been investigated. In this study, an *in vitro* culture system was employed to study the influence of copper on apoptosis and epigenetic factors in PC12 cells. When PC12 cells were exposed to copper, DNA damage was observed as DNA fragmentation. In addition, cytosolic cytochrome c levels were increased by copper treatment. These results suggested that copper induced apoptosis via an oxidative stress pathway. This was consistent with the observation that copper-induced apoptosis was enhanced by further oxidative stress induced by exposing cells to H_2O_2. In addition, the epigenetic factors were significantly increased in apoptotic cells following exposure to copper and oxidative stress.

Keywords: DNA methylation, Epigenetic, Sir2, Copper, Oxidative Stress

INTRODUCTION

Copper (Cu) is an essential trace element that requires careful homeostasis within mammalian cells to provide the necessary level, whilst avoiding the toxic effects associated with excess Cu. The induction of the formation of reactive oxygen species (ROS) played a major role on Cu toxicity in trout hepatocytes.[1] The concept of a lysosomal-mitochondrial axis of cell death induced by

oxidants has been described.[2] According to this concept, oxidative damage of lysosomes could result in released lysosomal enzymes acting on the mitochondria, where ROS formation would be enhanced and/or cytochrome c and other proapoptotic factors would be released.[3] Many related studies have produced data consistent with the above reports. For instance, dithiocarbamates (DCs) are sulfur-based metal chelators that are known to exert pro-oxidant and antioxidant effects in both cell-free and biological systems.[4] Low concentrations of N,N-diethyldithiocarbamate (DEDTC) induce apoptosis by raising the intracellular Cu level, triggering cytochrome c release and caspase activation via the formation of DEDTC-Cu complexes in human SH-SY5Y neuroblastoma cells.[5] However, the mechanisms underlying abnormal Cu metabolism, and the resultant oxidant status require further elucidation.

The term "epigenetic changes" refers to heritable alterations in gene expression that do not involve changes in the DNA sequence. Epigenetic changes mainly originate from DNA methylation, histone modification and non-coding RNAs, all of which can regulate gene expression patterns potentially leading to abnormal expression of multiple genes and inducing diseases.[6-9] DNA methylation is catalyzed by three major DNA methyltransferases (DNMTs).[10] DNMT1 maintains methylation status,[11] while DNMT3a and 3b are likely involved in *de novo* methylation.[12]

SIRT1 is a highly-conserved longevity-related gene which encodes sirtuin 1 (known as nicotinamide adenine dinucleotide-dependent deacetylase sirtuin-1), one of six members of SIRT gene family.[13] SIRT1 and SIRT2 are considered to play important roles in cell survival, differentiation, and metabolism.[14]

These epigenetic factors (DNMT1, DNMT3a, SIRT1 and SIRT2) can be influenced by various environmental factors. We have previously established a useful tool for assessing the risk of chemical toxicity by measuring the mRNA expresssion of the epigenetic factors in an *in vitro* cell culture system.[15] PC12 cells, a cell line derived from rat pheochromocytoma cells, provide a mature neuroendocrine cell model for studying diseases of the nervous and endocrine systems.[16,17] In our previous study, we showed that expression of DNMT3a, DNMT3b, and SIRT1 during serum deprivation-induced apoptosis in PC12 cells was influenced by an endocrine disrupter (diethyl phthalate).[15] However, the relationship between the effects of Cu on organisms, and epigenetic changes, is still unclear. In this study, PC12 cells were used to study the influence of Cu on apoptosis and epigenetic factors, and the effects of oxidative stress.

MATERIALS AND METHODS

Materials

PC12 cells were purchased from the American Type Culture Collection (Manassas, VA, USA). Dulbecco's modified Eagle's medium (DMEM) was obtained from Sigma-Aldrich (St. Louis, MO, USA). Fetal bovine serum (FBS) was bought from HyClone (Rockville, MD USA). The goat anti-mouse IgG biotinylated whole antibody from goat and enhanced chemiluminescence (ECL) western blotting detection reagents were from Amersham Pharmacia Biotech (Buckinghamshire, England). The monoclonal antibody against cytochrome c was purchased from BD Biosciences Pharmingen

(San Jose, CA, USA). SV Total RNA Isolation System, and Access RT-PCR Introductory System were purchased from Promega (Madison, WI, USA). The highly pure PCR template preparation kit was bought from Roche Diagnostics (Mannheim, Germany). Agilent RNA 6000 Nano Reagents and Agilent DNA 7500 Reagents were purchased from Agilent Technologies (Waldbronn, Germany). The Rotor-Gene SYBR Green RT-PCR Kit was purchased from Qiagen (Pleasanton, CA, USA). Cytochrome c Releasing Apoptosis Assay Kit was from the Medical & Biological Laboratory Co., Ltd (Nagoya, Japan). Other chemicals were of analytical reagent grade. $CuSO_4$ and H_2O_2 were dissolved in distilled water and using a sterilizing-grade filter.

Cell Culture

PC12 cells were maintained in 25 cm^2 flasks in DMEM supplemented with 10% FBS in a humidified incubator at 37°C and 5% CO_2. Inactivation of FBS was carried out at 56°C for 30 min prior to use. The cells were preincubated overnight, and then the medium was replaced with serum-containing DMEM with or without $CuSO_4$ (final $CuSO_4$ concentration: 200 or 500 μM) and H_2O_2 (final H_2O_2 concentration: 250 μM). Five μL of a 1000-fold concentration stock solution of $CuSO_4$ and/or H_2O_2 in distilled water were added to 5 mL cell medium. For the negative control, 5 μL of distilled water was added to the DMEM supplemented with 10% FBS.

Cell Viability

Cell viability was measured by trypan blue exclusion assay. PC12 cells were incubated in serum-

containing DMEM with $CuSO_4$ and/or H_2O_2 for 72 hr. After the incubation, cells were stained with 0.25% trypan blue solution in phosphate-buffered saline. The numbers of total cells and trypan blue-stained cells were then counted using an automated cell counter (Bio-Rad, USA). Cell viability was expressed as a percentage of the total cell number in each experiment. Each experiment was repeated four times.

Electrophoresis of Genomic DNA

PC12 cells were cultured in 5 mL DMEM containing 10% FBS with $CuSO_4$ and/or H_2O_2, or 5 μL distilled water (negative control) for 72 hr. After this incubation, cells were harvested using a cell scraper and centrifuged at 700 × g for 5 min to remove the supernatant. Genomic DNA was isolated from the cell pellet using a high pure PCR template preparation kit, according to the manufacturer's instructions. After RNAase incubation, ethanol precipitation was carried out. DNA (3-5 μg) was then subjected to electrophoresis on a 1.5% agarose gel. After electrophoresis, the DNA was visualized by staining with ethidium bromide for 15min, and the agarose gel was photographed with a ChemiDoc XRS (Bio-Rad, USA) under UV illumination.

Western Blotting

The PC12 cells treated with $CuSO_4$ and/or H_2O_2 were harvested by cell scraper, washed with ice-cold PBS and resuspended in 500 μL Cytosol Extraction Buffer Mix containing protease inhibitor cocktail and 1 mM DTT. The mixture was allowed to stand on ice for 10 min. For analysis of

cytochrome c, leaked cytochrome c in the cytosol was isolated using the Cytochrome c Releasing Apoptosis Assay Kit. The protein concentrations in each sample were measured by Bradford assay (Protein Assay, BIO-RAD, USA). The obtained solution was heated for 5 min at 100°C, and 0.1% BPB-glycerol was added.

Supernatant proteins (10 μg) was loaded and separated by SDS-PAGE (12.5% and 5%–20% e-PAGEL, ATTO, Japan), and the electrophoresed proteins were transferred onto nitrocellulose membranes using a semidry blotting system (type-AE6678, ATTO, Japan). The membranes were then incubated with a monoclonal antibody against cytochrome c (dilution; 1:200) in 40 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl and 1% blocking reagent for 30 min at 37°C. Anti-mouse IgG biotinylated was incubated with the membrane (dilution; 1:200). Finally, streptavidin-conjugated peroxidase (1:400, diluted in the same blocking buffer) was added and the membrane was incubated for 30 min at 37°C. Protein bands detected by the antibody were visualized using an enhanced chemiluminescence system, after washing the membrane with washing buffer. The images of the detected bands were analyzed using a ChemiDoc XRS (Bio-Rad, USA).

mRNA Expression Analysis by Real-time Reverse Transcription-polymerase Chain Reaction (Real-time RT-PCR)

Total RNA was prepared from PC12 cells cultured in serum-containing medium with 500 μM $CuSO_4$,

500 μM $CuSO_4$, and 250 μM H_2O_2 for 72 hr was prepared using the SV Total RNA Isolation kit. The PCR primers for DNMT1, DNMT3a, SIRT1, SIRT2, and β-actin were synthesized according to the DNA sequence as described previously.[15] The real-time RT-PCR analysis was carried out as described in our previous study.[18] Briefly, the RT reaction was carried out at 55°C for 10 min, and subsequently 40 PCR cycles were executed with an initial activation step of 95°C for 5 min, a denaturation of 95°C for 5 sec, and an annealing and extension step of 60°C for 10 sec. β-actin was chosen as an internal control. PCR products were real-time monitored using Rotor-Gene Q (USA) software. Real-time PCR specificity and identity were verified by melting curve analysis of the PCR products. Furthermore, to improve the accuracy of these RNA expression analyses, standard curves were always used to calculate the relative copy numbers of the target genes and β-actin using cycle threshold (Ct) values. Relative values of target gene expression in cells exposed to Cu and H_2O_2 were obtained from the ratio of target-genes copy numbers/β-actin, in comparison with that in cells that were not exposed to Cu and H_2O_2. These values were defined as the relative values of mRNA expression for each target gene.

Statistical Analyses

All values are presented as mean ± S.E.M. and treatment groups were compared using a one-way analysis of variance (ANOVA), followed by Student's t-test.

RESULTS

Cell Viability

To investigate whether Cu and H_2O_2 showed cell toxicity, cell viability was measured by trypan blue staining after exposure of PC12 cells to 200 or 500 μM $CuSO_4$, with or without250 μM H_2O_2, for 72 hr. As shown in Figure 1a, significant decreases in cell viability were observed in the cells exposed to 200 and 500 μM $CuSO_4$ in comparison with control cells. In addition, the cell viability following exposure to both Cu and H_2O_2 treatment was significantly lower than that observed with exposure to Cu alone. These results demonstrated that Cu toxicity was enhanced in the presence of H_2O_2.

Detection of DNA Fragmentation by Agarose Gel Electrophoresis

It has been reported that apoptosis can be induced by serum deprivation in PC12 cells.[19] Apoptosis is frequently accompanied by multiple cleavage of DNA into 180–200 base pair fragments. These oligonucleosomal fragments can be visualized as a characteristic DNA ladder, following agarose gel electrophoresis. DNA fragmentation was observed in PC12 cells cultured in serum-containing medium with 500 μM $CuSO_4$ (Figure 1b). However, DNA laddering was low in the presence of Cu and H_2O_2.

Cytochrome c Release Detected by Western Blotting Analyses

To investigate whether Cu affected apoptotic pathways via oxidative stress, western blotting analyses

using lysates from PC12 cells treated with Cu and/or H_2O_2 were performed using an anti-cytochrome c monoclonal antibody. Apoptosis induced by oxidative stress is generally associated with release of mitochondrial cytochrome c into the cytosol. After treatment with Cu, cytosolic cytochrome c levels in PC12 cells were obviously increased in comparison with those in cells not treated with Cu (Figure 2). In addition, cytochrome c release in PC12 cells was increased by the addition of H_2O_2, an oxidant, to Cu. It was suggested that Cu treatment caused oxidative stress, and H_2O_2 enhanced the oxidative stress caused by Cu treatment.

mRNA Expression of Epigenetic Factors in PC12 Cells Exposed to Cu and H_2O_2

To examine whether expression of epigenetic factors was altered by exposure to $CuSO_4$ and H_2O_2, DNMT1, DNMT3a, SIRT1 and SIRT2 were measured using real-time RT-PCR analysis.

As shown in Figure 3, there were significant differences between the levels of DNMT1 (Figure 3a) and SIRT1 (Figure 3b) mRNA in the PC12 cells cultured in the serum-containing medium with 500 μM $CuSO_4$. DNMT1 mRNA levels in PC12 cells also tended to increase when 250 μM H_2O_2 was added to Cu-containing medium (Figure 3a). When PC12 cells were exposed to 500 μM $CuSO_4$ and 250 μM H_2O_2, the relative expression level for SIRT1 had a wider variance than that of the control. Although no significant differences were observed, PC12 cells exposed to 500 μM $CuSO_4$ showed higher levels of DNMT3a (Figure 3c) and SIRT2 (Figure 3d) mRNAs than control cells. When 250 μM H_2O_2 was added to Cu-containing medium, expression of both DNMT3a and SIRT2 in PC12 cells was significantly increased as compared with that of control cells. There were no

significant H_2O_2-dependent differences in DNMT1, DNMT3a, SIRT1 or SIRT2 mRNA expression in Cu-treated PC12 cells. These findings indicated that both DNMT1 and SIRT1 expression may have been upregulated by induction of apoptosis by Cu in PC12 cells. It was interesting that increased DNMT1, DNMT3a, SIRT1 and SIRT2 expression were observed in PC12 cells simultaneously exposed to 500 μM $CuSO_4$ and 250 μM H_2O_2 showed, because all of these genes may be involved in a Cu-induced apoptotic pathway.

DISCUSSION

The accumulation of Cu was reported to induce reversible oxidative stress in hepatocytes, leading to cytotoxicity with oxidative damage, and finally resulting in apoptosis due to the enhancement of free radical levels.[20] From the results in this study, it was indicated that gene expression of DNMT1 and SIRT1 in PC12 cells was activated by Cu (Figures 3a and b). In addition, this activation was amplified by the addition of H_2O_2 (Figure 3). Cu has the redox properties of a transition metal and has been reported to promote lipid peroxidation probably due to hemolytic scission of H_2O_2 and of endogenous hydroperoxides (ROOH), the generating hydroxyl (HO•) and alcoxyl (RO•) radicals.[21] These findings were supported by the results presented here. Previously, DNMT3a expression was reported to be significantly decreased following serum-deprivation in PC12 cells which induced apoptosis that was dependent on oxidative stress.[15] The reason for this conflict between previous and present results is still unclear.

On the other hand, serum deprivation directly affects the essential factors. For this reason, *de novo*

synthesis ability of DNA methylation in PC12 cells may depend on the presence of essential factors. DNMT3a gene expression in PC12 cells was decreased by serum deprivation and increased by Cu treatment, although both actions induced apoptosis involving oxidative stress.

H_2O_2 accelerated the oxidative stress caused by Cu. The presence of Cu, H_2O_2 and ascorbic acid in cells can trigger a Fenton-type reaction.[22] As shown in Figure 1a and Figure 2, the cell viability and released cytochrome c levels were significantly decreased in PC12 cells treated with Cu and H_2O_2, compared with those treated with Cu alone. However, DNA ladder was not observed in the PC12 cells exposed to Cu and H_2O_2 (Figure 1b). This may have been due to complete DNA digestion in the presence of Cu and H_2O_2, with the smaller fragments running out of the bottom of the agarose gel, or decreased DNA synthesis in these cells due to the severe damage caused by Cu and H_2O_2.

As shown in Figure 3, DNMT1, DNMT3a, SIRT1 and SIRT2 gene expression in PC12 cells treated with Cu and H_2O_2 had a tendency to increase. Previous reports have indicated that SIRT1 expression decreased under oxidative stress induced by H_2O_2.[23-25] In addition, resveratrol enhanced SIRT1 expression via p53 deacetylation to depress oxidative stress.[25] p53 expression after diethyl phthalate (DEP) treatment was increased in PC12 cells, which protects the cell from DEP toxicity due to the oxidative stress.[15] The increased SIRT1 expression identified in the present study may contribute to protecting cells against Cu and H_2O_2 induced-oxidative stress..

Furthermore, it has been reported that SIRT2 expression was increased by treatment with H_2O_2.[26] Consistent with this, the present study found that SIRT2 was significantly increased by treatment of PC12 cells with Cu and H2O2. Under oxidative stress, the activity and expression of DNMT1 and 3a

were reportedly increased.[27] In particular, arsenic-induced oxidative stress was associated with decreased DNMT1 and DNMT3a expression.[27] However, in this study, DNMT1 gene expression in PC12 cells treated with Cu was significantly increased, as compared with control. The reason for this discrepancy is unclear, but may relate to the activation of different oxidative stress pathways by arsenic and Cu. On the other hand, analysis of H_2O_2-resistant cells (OC-14) showed increased DNMT1 expression.[28]

In conclusion, DNA damage related to oxidative stress was observed in PC12 cells exposed to Cu and H_2O_2. Under conditions of oxidative stress in PC12 cells, the expression of epigenetic factors (DNMT1, DNMT3a, SIRT1, and SIRT2) tended to be upregulated. These results indicated that changes in epigenetic factors could be used as assessment tools for oxidative dysfunction in these cells. Further investigation will be needed to clarify the mechanisms regulating expression of these epigenetic factors in detail.

ACKNOWLEDGMENTS

This research was partly supported by Grants-in-Aid from the Japan Society for the Promotion of Science (No. 23655139 for Kurasaki).

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FIGURE CAPTIONS

Figure 1. Cell viability (a) and DNA agarose gel electrophoresis (b) of PC12 cells treated with 0-500 μM Cu and 250 μM H_2O_2 for 72 hr. Error bars indicate S.E.M. (n=4). *: there are significant differences in cell viability between control cells and each treatment cell ($p<0.01$). a, b, c, d: significant difference between the same letter ($p<0.05$).

Figure 2. Cytochrome c levels in PC12 cells with 0–500 μM Cu and 250 μM H_2O_2. *: there are significant differences in cell viability between control cells and each treatment cell ($p<0.05$). a, b, c, d: significant difference between the same letter ($p<0.05$)

Figure 3. mRNA expression of epigenetic factors determined by real-time RT-PCR for DNMT1 (a), SIRT1 (b), DNMT3a (c) and SIRT2 (d) in PC12 cells treated with and without $CuSO_4$ and H_2O_2 for 72 hr. Values are mean ± S.E.M. (n = 6). DNMT, DNA methyltransferase; SIRT, Sirtuin; $^{a, b}$ $P<0.05$ compared with the same letter.

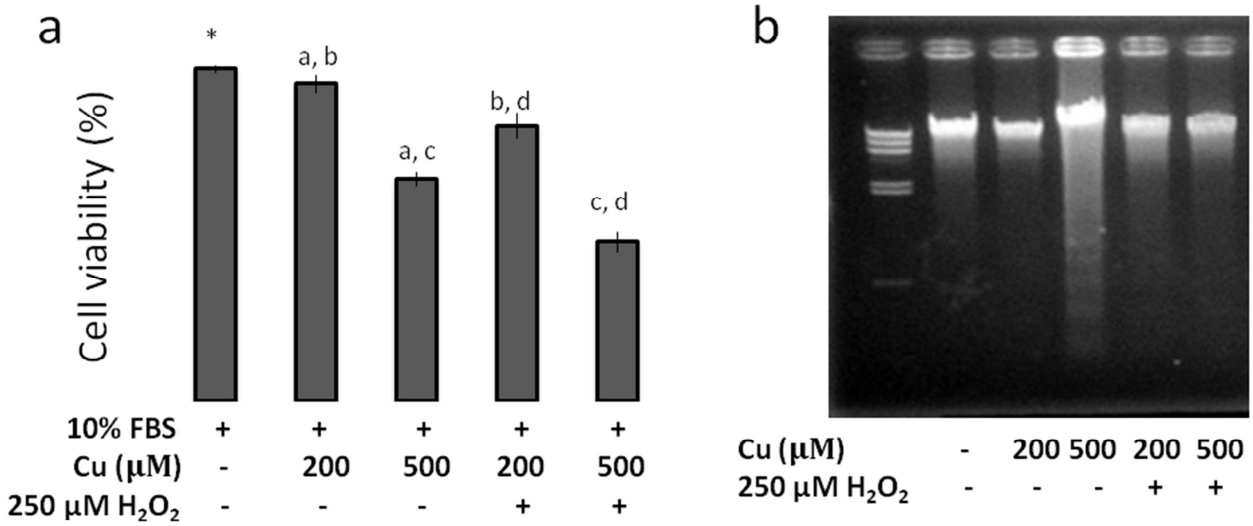
a
Cell viability (%)
*
a, b
a, c
b, d
c, d
10% FBS + + + + +
Cu (μM) - 200 500 200 500
250 μM H2O2 - - - + +
b
Cu (μM) - 200 500 200 500
250 μM H2O2 - - - + +

Fig. 1

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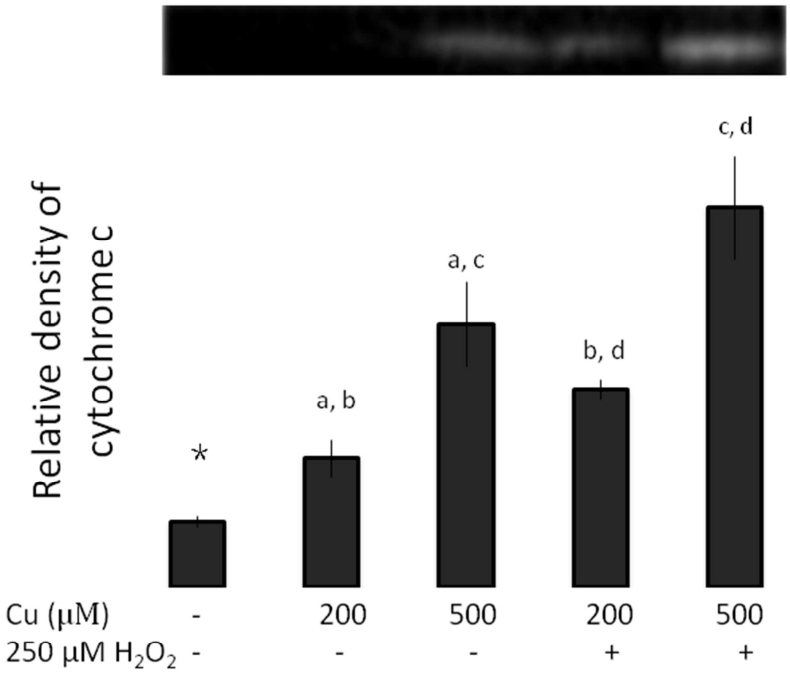
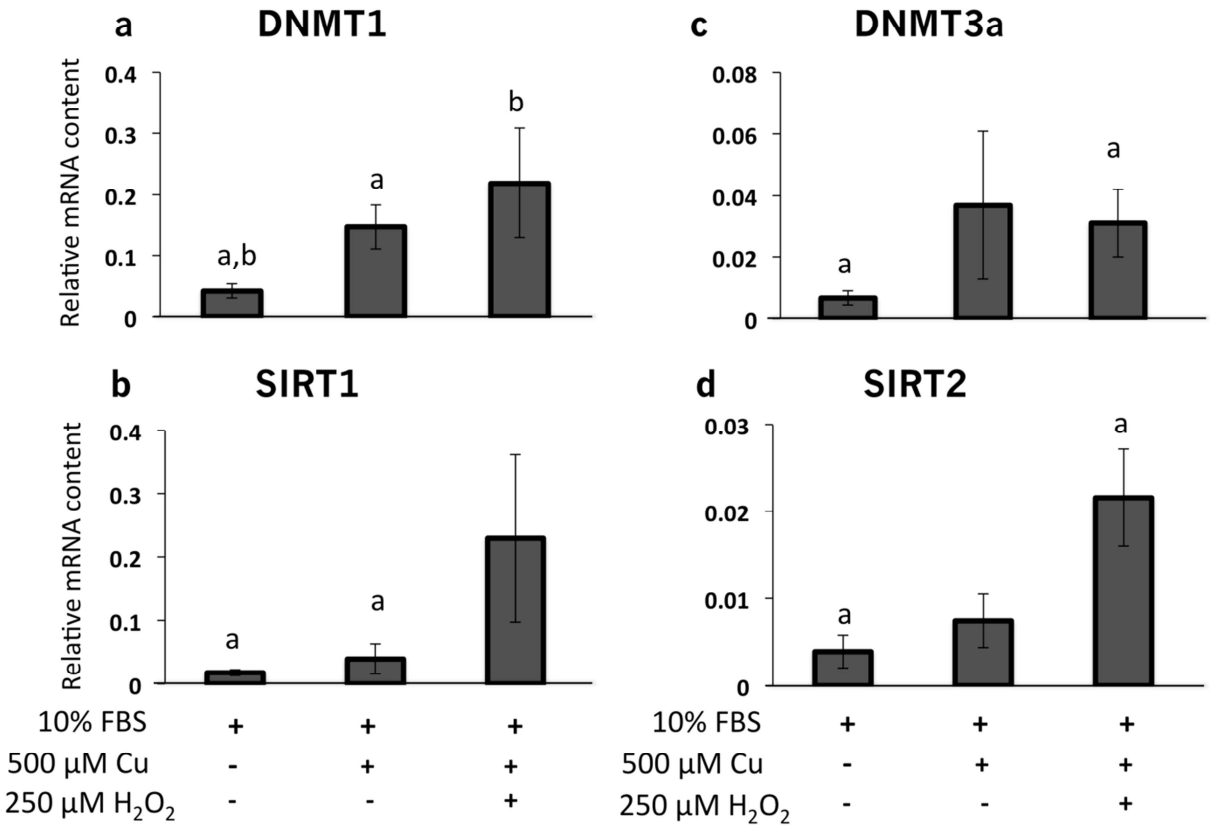

Relative density of cytochrome c
*
a, b
a, c
b, d
c, d
Cu (μM) - 200 500 200 500
250 μM H2O2 - - - + +

Fig. 2

355


a
DNMT1
Relative mRNA content
0.4
0.3
0.2
0.1
0
a,b
a
b
c
DNMT3a
0.08
0.06
0.04
0.02
0
a
a
b
SIRT1
Relative mRNA content
0.4
0.3
0.2
0.1
0
a
a
d
SIRT2
0.03
0.02
0.01
0
a
a
10% FBS + + +
500 µM Cu - + +
250 µM H2O2 - - +
10% FBS + + +
500 µM Cu - + +
250 µM H2O2 - - +

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357 Fig. 3