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Up-Regulation of Heme Biosynthesis during Differentiation of Neuro2a Cells

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Heme is an iron-containing tetrapyrrole molecule that functions as a prosthetic group for proteins such as mitochondrial respiratory enzymes. Several studies have suggested that heme has essential functions in the construction and maintenance of the nervous system. In this study, the contents of three biologically important forms of heme (types *a*, *b*, and *c*) and the expression of heme biosynthetic enzymes were examined in differentiating Neuro2a cells. During neuronal differentiation, there were increases in the cellular heme levels and increases in the mRNA levels for the rate-limiting enzymes of heme biosynthesis, such as aminolevulinic acid synthase (ALAS; EC 2.3.1.37) and coproporphyrinogen oxidase (EC 1.3.3.3). With respect to heme contents, heme *b* increased in the late phase of differentiation, but no apparent increase in heme *a* or *b* was observed in the early phase. In contrast, heme *c* (cytochrome *c*) markedly increased during the early phase of differentiation. This change preceded the increase in heme *b* and the up-regulation of the mRNA levels for heme biosynthetic enzymes. This study suggests the up-regulation of heme biosynthesis and differential regulation of the heme *a*, *b*, and *c* levels during neuronal differentiation.

Key words: cytochrome *c*, differentiation, heme, mitochondria, neuron.

Abbreviations: ALAS, 5-aminolevulinic acid synthase; USP, upstream primer; DSP, downstream primer.

Heme is an essential prosthetic group of mitochondrial respiratory enzymes and globin proteins, and several proteins are known to have regulatory heme-binding domains (*1*, *2*). Heme is required for the construction and maintenance of the nervous system, and altered heme metabolism is observed in patients with Alzheimer's disease. In particular, mitochondrial cytochrome *c* oxidase (complex IV; EC 1.9.3.1), which is a heme-containing enzyme, is markedly reduced in Alzheimer's disease (*3–6*). Heme has been shown to inhibit the *in vitro* aggregation of amyloid β protein and to protect neuronal cells from its toxic effects (*7*). Also, some heme proteins, such as neuroglobin and neuronal nitric oxide synthase (EC 1.14.13.39) are highly expressed in neuronal cells, and heme appears to be essential for their growth (*8*, *9*). In fact, the addition of heme to the culture medium induces the differentiation of neuroblastoma cells (*10*), and neuronal differentiation does not proceed when precursor cells are exposed to succinyl-acetone, an inhibitor of heme biosynthesis (*11–13*). Collectively, these studies indicate that heme is essential for the function of neurons. However, how heme synthesis is regulated during the course of neurogenesis has not been determined.

There are three biologically important types of heme, types *a*, *b* and *c* (Fig. 2b), which differ in their porphyrin ring structures (*14*). Heme *b* is the product of ferrochelatase (EC 4.99.1.1), the terminal enzyme of the heme synthetic pathway, whereas hemes *a* and *c* are formed through modification of heme *b*. Heme *b*, the most common form of heme, is the prosthetic group of proteins such as globins and is also present in mitochondrial respiratory complexes. Heme *c* differs from heme *b* (or protoheme) in that its two vinyl side chains are covalently bound to the protein itself. Examples of proteins containing a *c*-type heme include cytochromes *c* and c_1. In contrast to that of heme *b*, the methyl side chain of heme *a* is oxidized into a formyl group, and one of the vinyl side chains (on C2) is replaced by an isoprenoid (farnesyl) chain. The only known protein to contain heme *a* is complex IV of the mitochondrial respiratory system, which contains two noncovalently bound heme *a* groups. All three forms of heme are essential components of the mitochondrial respiratory system and a variety of other systems, such those regarding gas transport and storage by globins.

In this study, we analyzed the expression of mRNAs for heme synthetic enzymes during retinoic acid-induced differentiation of Neuro2a cells. We also examined the alteration in the contents of the three types of heme (*a*, *b*, and *c*) in these cells.

MATERIALS AND METHODS

Cell Culture—Neuro2a cells were cultured in Dulbecco's modified Eagle's medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% (v/v) fetal bovine serum (Gibco BRL Life Technologies, Paisley, Scotland), 100 U/ml penicillin, 100 μg/ml streptomycin, and 292 μg/ml glutamine at 37°C in a humidified 5% (v/v) CO_2 incubator. The cells were seeded at 1.0×10^6 and then incubated for 24 h in 90-mm-diameter plastic dishes. Then induction of

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differentiation was performed as described (*15–17*). Cells were differentiated with 20 μM retinoic acid in Dulbecco's modified Eagle's medium containing 2% (v/v) fetal bovine serum for 0–96 h (0 h means untreated control, cultured without retinoic acid). The culture medium was routinely changed every 2 days.

Quantitative Real-Time PCR—Total RNA was isolated from cells using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol. RNA was reverse-transcribed, and complementary DNAs were synthesized using an oligo (dT) primer. Real-time PCR was performed using a LC real-time PCR apparatus (Roche Diagnostics, Mannheim, Germany) and a Quantitect SYBR-Green RT-PCR Kit (Qiagen, Hilden, Germany) in a 20 μl volume containing 0.5 μM of each upstream primer (USP) and downstream primer (DSP) according to the manufacturer's instructions. The primers used were as follows: β-actin-USP, 5′-tggaatcctgtggcatccatgaaac-3′; β-actin-DSP, 5′-taaaacgcagctcagtaacagtccg-3′; ferrochelatase-USP, 5′-ccactgtccacaaagtc-3′; ferrochelatase-DSP, 5′-gatagcctcatctgtctg-3′; ALAS-1-USP, 5′-gtcaagcttctgaggc-3′; ALAS-1-DSP, 5′-cctggtcatcaactc-3′; 5-aminolevulinic acid dehydratase (EC 4.2.1.24)-USP, 5′-ggtgaagccgggattgc-3′; 5-aminolevulinic acid dehydratase-DSP, 5′-ggaaggccgtcatggt-3′; porphobilinogen deaminase (EC 2.5.1.61,)-USP, 5′-ccgtagcagtgcatacagtg-3′; porphobilinogen deaminase-USP, 5′-ctggatggtggcctgcatag-3′; protoporphyrinogen oxidase (EC 1.3.3.4)-USP, 5′-ccattccagcttcagagc-3′; protoporphyrinogen oxidase-DSP, 5′-cagacaagctcctcggtac-3′; coproporphyrinogen oxidase (EC 1.3.3.3)-USP, 5′-ctccaggatccaggatc-3′; coproporphyrinogen oxidase-DSP, 5′-cctttggatggcgcaac-3′; uroporphyrinogen cosynthase (EC 4.2.1.75)-USP, 5′-ctatcagacagttcc-3′; uroporphyrinogen cosynthase-DSP, 5′-cgcctgaatatactc-3′; uroporphyrinogen decarboxylase (EC 4.1.1.37)-USP, 5′-ggctatgaggtagttggac-3′; and uroporphyrinogen decarboxylase-DSP, 5′-gatgcatacaaggcacagg-3′. The PCR was carried out as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of 10 s at 95°C, 10 s at 60°C, and 20 s at 72°C. The mRNA levels were normalized as to the level of β-actin mRNA.

Extraction and Separation of Hemes—Extraction and separation of hemes were performed as described previously (*18*) with slight modifications. Cells and mitochondria were solubilized with 1% (v/v) Tween20 and then hemes were extracted with acetone containing 2.5% (v/v) HCl. The mixture was vortexed, centrifuged for 5 min at $1,400 \times g$ and then mixed with 50% (v/v) acetonitrile. Insoluble material was removed by a second centrifugation. Next, the extracts were adjusted to approximately pH 3.5 with 1.65 M ammonium hydroxide and then clarified by centrifugation. The extracts were applied to a 2×50 mm Luna 3 micron C18(2) column (Phenomenex, CA, Torrance, USA). Hemes were eluted at the flow rate of 0.2 ml/min using a gradient of 10–50% (v/v) acetonitrile for the first 1 ml, followed by a linear gradient of 50–75% (v/v) acetonitrile for the subsequent 1 ml. All gradient solutions contained 0.05% (v/v) trifluoroacetic acid. The elution and identification of heme compounds were performed at 400 nm. Heme *b* standard was purchased from Sigma. Heme-*a* was prepared from partially purified complex IV from bovine heart mitochondria (*19*) and used as a standard. Hemes *a* and *b* were quantified by comparing each peak area with that of a known amount of the standard. Data are shown as concentrations of heme per mg protein.

Sodium Dodecyl Sulphate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Heme/Protein Staining—Mitochondria were prepared using an ApoAlert cell fractionation kit (BD Biosciences, CA, USA) following the protocol in the user manual. The isolated mitochondria were incubated for 15 min at 42°C in 124 mM Tris, pH 7.0, 20% (v/v) glycerol, and 4.6% (w/v) SDS, but without β-mercaptoethanol. Proteins were separated by electrophoresis on 10–20% (w/v) polyacrylamide gradient gels (Daiichi, Tokyo, Japan) and then transferred to nitrocellulose filters (BA 85 Schleicher & Schuell, Dassel, Germany) as described by Towbin *et al*. (*20*).

To determine the content of heme *c*, the detection procedure for *c*-type cytochrome, which is based on the intrinsic peroxidase activity of the heme groups of denatured *c*-type cytochromes, reported by Vargas *et al*. (*21*) was used. *C*-type cytochromes, denatured and separated by SDS-PAGE, were detected directly using an enhanced chemiluminescence (ECL) system (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer's instructions.

After protein transfer, the nitrocellulose membranes were further used for the detection of cytochrome *c* and VDAC proteins with antibodies as mentioned below. Detection of proteins by means of Western blotting was performed as follows: after blocking with 5% (w/v) skim milk in Tris-buffered saline plus Tween 20 [TBST: 50 mM Tris, pH 7.5, 0.15 M NaCl, 0.05% (w/v) Tween 20], the membranes were incubated with primary antibodies in TBST containing 2% (w/v) skim milk, after which they were incubated with secondary antibodies in TBST containing 2% (w/v) skim milk. Detection was carried out with an alkaline phosphatase system. Densitometric intensity was quantified with NIH image 1.62.

To determine the cytochrome *c* content of whole cells, total cell lysates were prepared by washing the cells twice with phosphate-buffered saline and then homogenizing them in cooled lysis buffer (10 mM EDTA, 1% Tween20, 10 mM Tris-HCl, pH 7.5) supplemented with a protease inhibitor cocktail for general use (Sigma-Aldrich). After centrifugation to remove insoluble material, equivalent amounts of protein were denatured in sample buffer [60.5 mM Tris-HCl, pH 6.8, 2% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 7.9% (v/v) glycerol, 0.05% (w/v) bromophenol blue] and then heated at 94°C for 5 min. 20 μg of protein was loaded and separated by electrophoresis on a 15% (w/v) polyacrylamide gel. The transfer of proteins to nitrocellulose filters and reaction with antibodies were performed as described above. The detection of proteins of total cell lysate was carried out with ECL reagents (Amersham Biosciences).

The antibodies used were as follows: rabbit polyclonal cytochrome *c* antibodies supplied in the ApoAlert cell fractionation kit (1:1,000; BD Biosciences), and rabbit polyclinal VDAC antibodies (1:5,000; Abcam, Cambridge, MA) and mouse monoclonal β-actin antibodies (1:5,000; clone AC-15, SIGMA, St Louis, MO, USA) as primary antibodies, and anti-rabbit IgG alkaline phosphatase conjugate (1:5,000; Bio-Rad Laboratories, CA, USA) as the secondary antibodies for the detection of mitochondrial proteins with an alkaline phosphatase system.

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Data Analysis—Statistical analyses were performed using one-way ANOVA coupled with Tukey's pairwise comparisons. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

The Effects of Heme Synthesis Inhibitors on the Differentiation of Neuro2a—In this study we used Neuro2a cells, because they differentiate on the addition of 20 μM retinoic acid (*15–17*) and detailed information during differentiation after retinoic acid treatment (96 h) has been presented (*17*). As shown in Fig. 1a, morphologic changes such as neurite sprouting and extension after retinoic acid exposure were observed during this period (0–96 h).

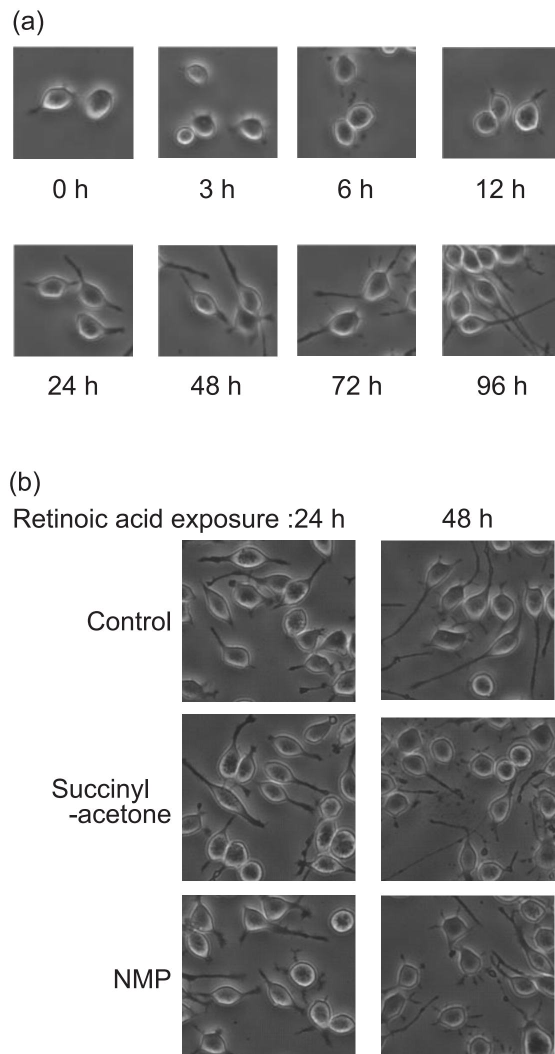

Fig. 1. **a: Retinoic acid–induced differentiation of Neuro2a cells.** The differentiation of Neuro2a cells was induced with 20 μM retinoic acid in Dulbecco's modified Eagle's medium containing 2% (v/v) fetal bovine serum. 0 h means before retinoic acid treatment, that is cells were proliferating, and was considered as a control. Cells at 3 h, 6 h, 12 h, 24 h, 48 h, 72 h and 96 h after the onset of retinoic acid exposure were analyzed to study the differentiation process. **b: The effect of heme biosynthetic inhibitors on retinoic acid–induced differentiation of Neuro2a cells.** Neuro2a cells were either treated with 20 μM retinoic acid alone (control), 20 μM retinoic acid and 1 mM succinyl-acetone (succinyl-acetone), or 20 μM retinoic acid and 10 μM NMP (NMP) for 24 h or 48 h.

As described previously, NGF-induced differentiation of PC12 cells and SHSY5Ycells does not proceed when the precursor cells are exposed to succinyl-acetone or *N*-methylprotoporphyrin IX (NMP), an inhibitor of heme biosynthesis (*11*, *12*, *22*). To determine whether or not the differentiation of Neuro2a cells was also interfered with heme synthesis inhibitors, the differentiation of Neuro2a cells was compared in the presence and absence of heme biosynthesis inhibitors (Fig. 1b).

As shown in Fig. 1a, neurite sprouting and extension of control cells, which had been treated under normal differentiation conditions (given in materials and methods), were observed within 24 h and further neurite extension was observed thereafter (48, 72, 96 h). In the presence of 1 mM succinyl-acetone or 10 μM NMP, neurite sprouting and extension were observed to 24 h. However, further extension or maintenance of the neurites did not continue to 48 h after the onset of differentiation induction. Under normal growth conditions (without retinoic acid), no difference was observed morphologically between the conditions with and without heme synthesis inhibitors (data not shown).

Expression of mRNAs for Heme Biosynthetic Enzymes during Neuronal Differentiation—To study heme biosynthetic activity during neuronal differentiation, we examined the mRNA levels for eight heme biosynthetic genes (Fig. 2). The mRNA levels of most of the heme biosynthetic enzymes increased during differentiation (Fig. 3). No change was observed in the mRNA level for porphobilinogen deaminase. ALAS, coproporphyrinogen oxidase, and protoporphyrinogen oxidase were more up-regulated than the other enzymes. Increases in the mRNA levels were observed within 48 h, and no further increases were observed thereafter.

Changes in the Heme a *and* b *Contents during Neuronal Differentiation*—To examine the changes in cellular heme contents, non-covalently bound heme (types *a* and *b*) was extracted from cells exposed to retinoic acid for 0–96 h and then quantitatively analyzed by HPLC (Fig. 4). As shown in Fig. 4a and b, in the early phase of differentiation (within 6 h), the heme *a* and *b* contents slightly decreased. A gradual increase in the cellular heme *b* level was observed between 12 and 48 h, and a clear increase was evident after 72 h. In contrast, the heme *a* content did not change significantly during retinoic acid–induced differentiation.

Next we examined the mitochondrial heme content to determine whether or not the change in the cellular heme *b* content was due to the alteration of the amount of mitochondrial heme proteins, such as those of respiratory enzymes (Fig. 4, c and d). Mitochondrial heme *b* also decreased in the early phase of differentiation. However, a low level of heme *b* continued longer than the cellular level (to 24 h) and, unlike the cellular heme *b* level, mitochondrial heme *b* did not reach a higher level than that of 0 h even in the later phase (72, 96 h), although recovery was observed from 48 to 96 h after the onset of differentiation induction. The mitochondrial heme *a* level did not change significantly, although a slight decrease and increase were observed in the early phase and later phase of differentiation, respectively.

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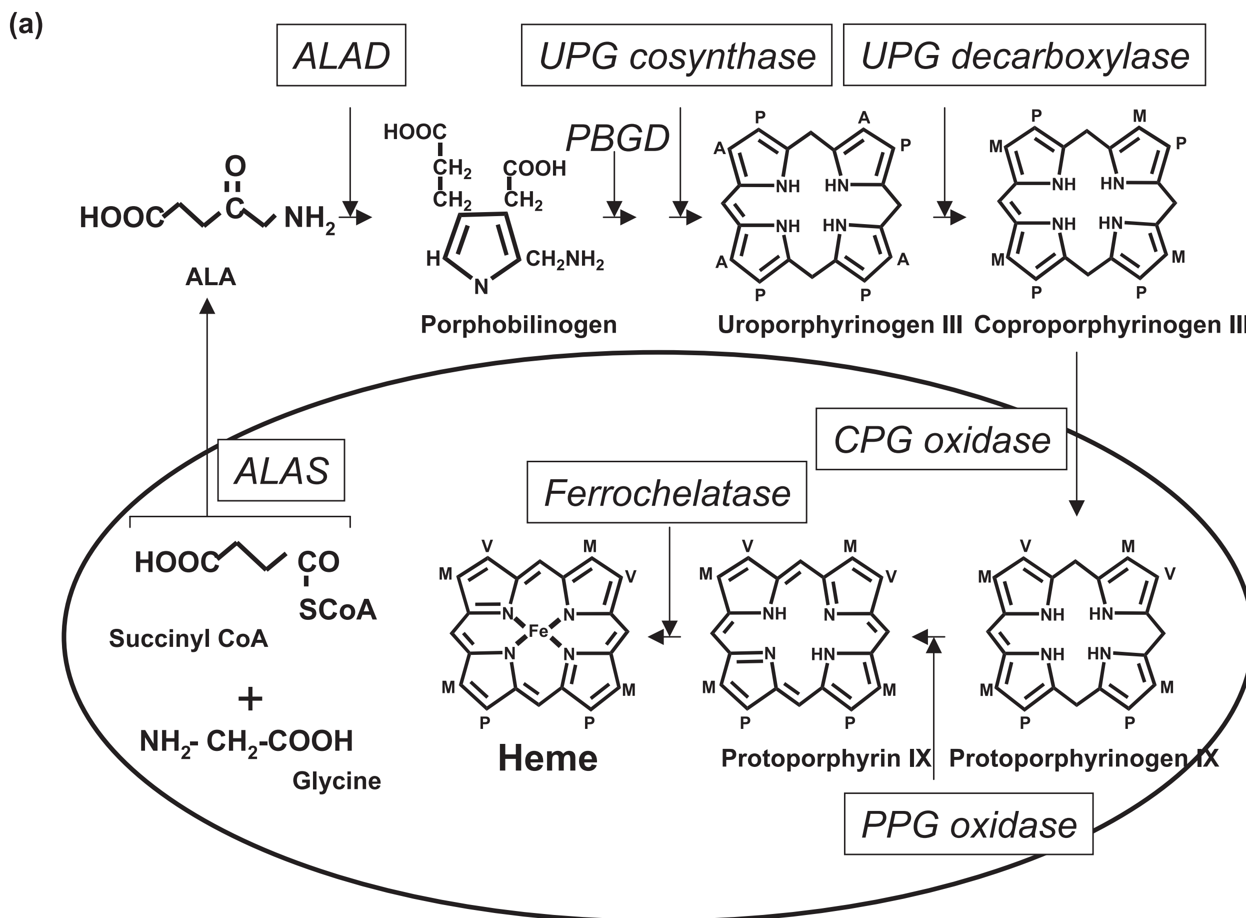

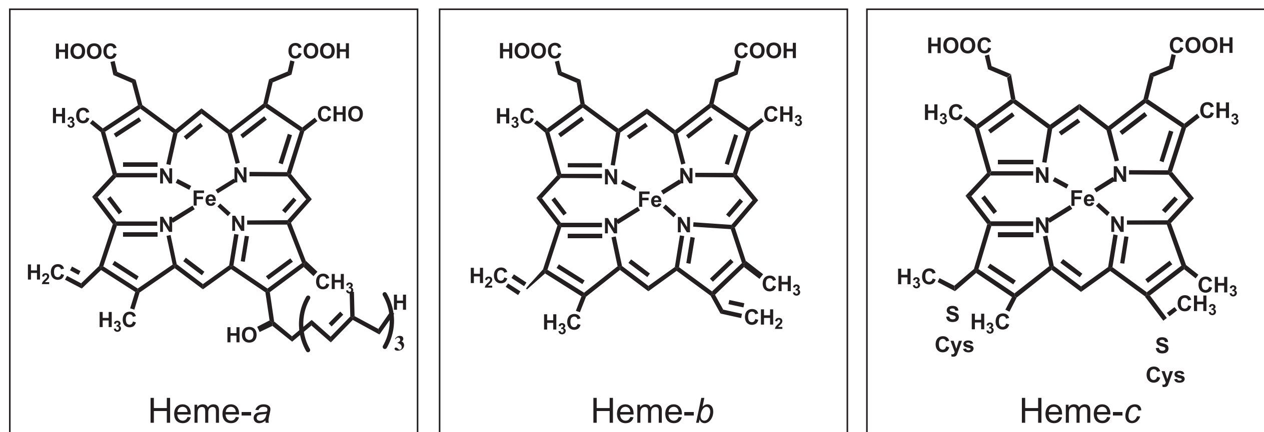

Fig. 2. **a: Heme biosynthetic pathway in mammals.** The enzymes are compartmentalized between the mitochondria and cytosol, with the first enzyme (ALAS) and the last three enzymes located in the mitochondria. ALAS catalyses the conversion of succinyl CoA and glycine to ALA. The last enzyme, ferrochelatase, catalyses the insertion of a ferrous ion into protoporphyrin IX to form heme. ALA, 5-aminolevulinic acid; PBGD, porphobilinogen deaminase; ALAD, 5-aminolevulinic acid dehydratase; UPG, uroporphyrinogen; CPG, coproporphyrinogen; PPG, protoporphyrinogen; A, acetyl; M, methyl; V, vinyl; P, propionyl. **b: The structures of heme *a*, *b*, and *c*.** The structures of the three heme types are shown. Cys in the heme *c* structure are the cystein residues of cytochrome *c* proteins.

Change in Mitochondrial Heme c *during Differentiation*—In contrast to hemes *a* and *b*, heme *c* cannot be extracted with acetone-HCl because it is covalently attached to the protein. Therefore, heme *c* was detected using the method specific for *c*-type cytochrome involving ECL as described under Materials and methods. Since heme *c* (cytochrome *c* and c_1) is localized in mitochondria and the sensitivity of the heme staining method is too low

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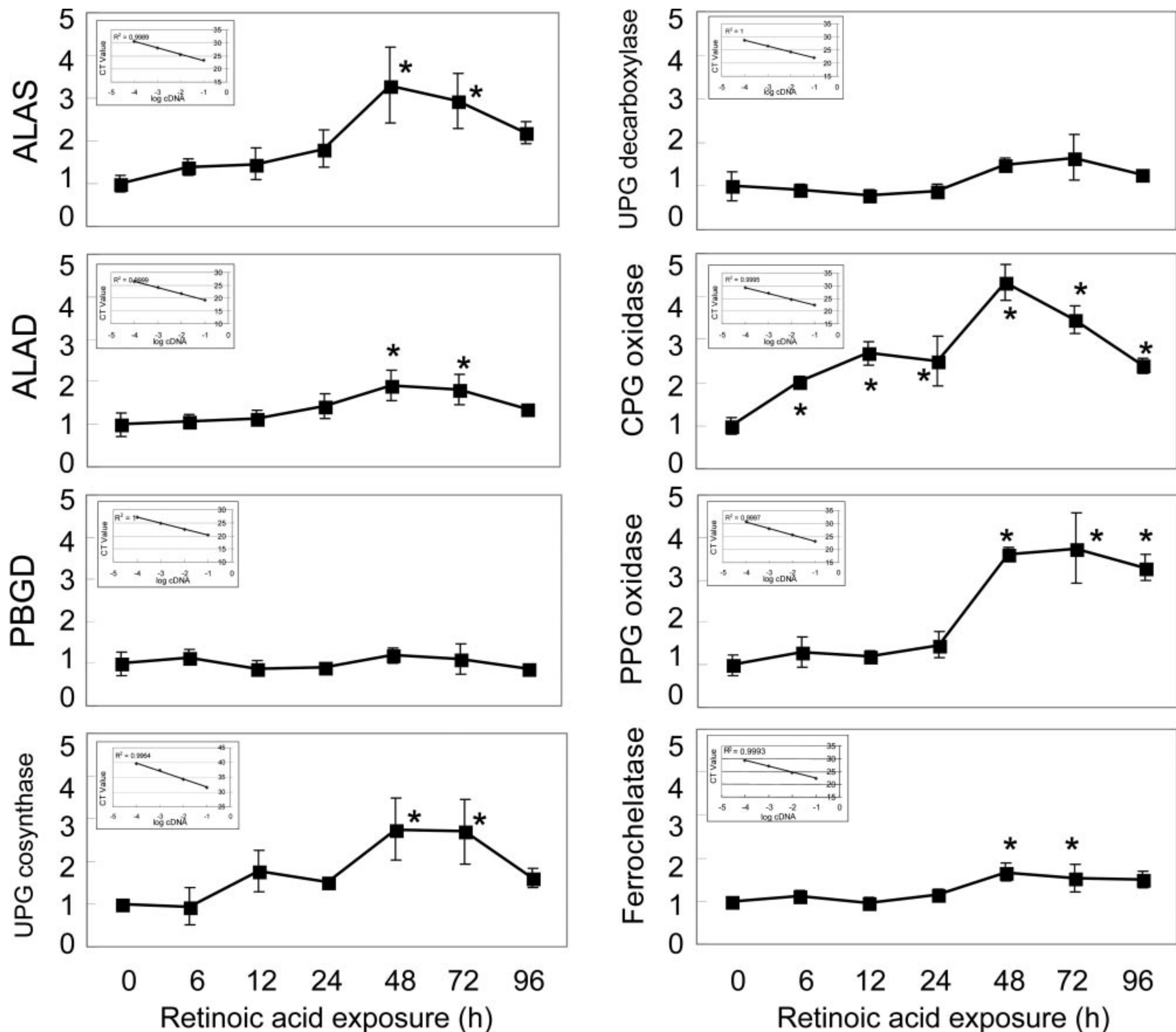

Fig. 3. **mRNA levels of heme biosynthetic enzymes.** Total RNA was extracted from differentiating cells, and cDNA was prepared by reverse transcription. Determination of mRNA levels was performed by real-time PCR. The mRNA levels were normalized as to the levels of β-actin mRNA. The results represent the means ± SD (n = 3). A representative standard curve for each enzyme is shown in the inset. $^{*}P < 0.05$ *vs.* 0 h, $^{**}P < 0.05$ *vs.* 6 h (ANOVA followed by Tukey's pairwise comparisons).

to detect cellular heme c, we prepared mitochondria from differentiating cells for analysis.

The only heme c band observed after ECL staining was one corresponding to a molecular mass of 12 kDa, which corresponds to cytochrome c (Fig. 5a, lower panel). This was confirmed by Western blotting with cytochrome c specific antibodies (Fig. 5a, upper panel). The level of mitochondrial heme c (cytochrome c) increased during differentiation, whereas the content of control protein (VDAC) did not change. It is of interest to note that cytochrome c rapidly increased during the early phase of differentiation (within 12 h of retinoic acid exposure) in contrast to hemes a and b. A band corresponding to cytochrome c_1 (27 kDa) was not obsreved. This may be due to the low content of cytochrome c_1 in the mitochondria (*23*).

We next examined the level of cellular cytochrome c protein (Fig. 5b). In contrast to the level of mature cytochrome c protein in mitochondria, the level of cytochrome c polypeptide in the total cell lysate did not increase significantly in the early phase of differentiation (0–12 h), although an obvious increase was observed in the later phase (72–96 h).

DISCUSSION

In the current study, we examined the changes in the heme contents and mRNA levels of heme biosynthetic enzymes to determine the significance of heme synthesis during neuronal differentiation. For these investigations, we used retinoic acid-treated Neuro2a cells as a model of neuronal differentiation.

Neuro2a cells can be induced to differentiate into neuron-like cells with neurites by the addition of retinoic acid (*15–17*). A morphologic change and an increase in acetylcholine-esterase activity, which is a marker for

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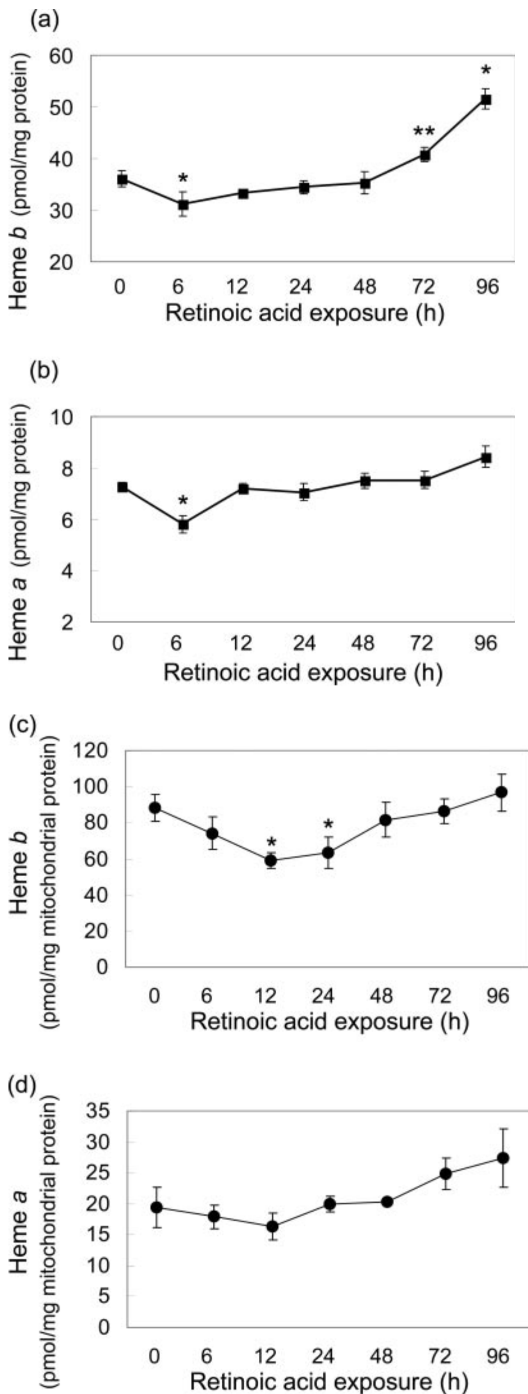

Fig. 4. **Levels of hemes *b* and *a* in differentiating Neuro2a cells.** The heme *b* (a) and heme *a* (b) contents of total cells, and heme *b* (c) and heme *a* (d) contents of mitochondria were determined. The values are normalized as to protein (mg) and the means ± SD (n = 3) are shown. *$P < 0.05$ *vs.* 0 h, **$P < 0.05$ *vs.* 6 h (ANOVA followed by Tukey's pairwise comparisons).

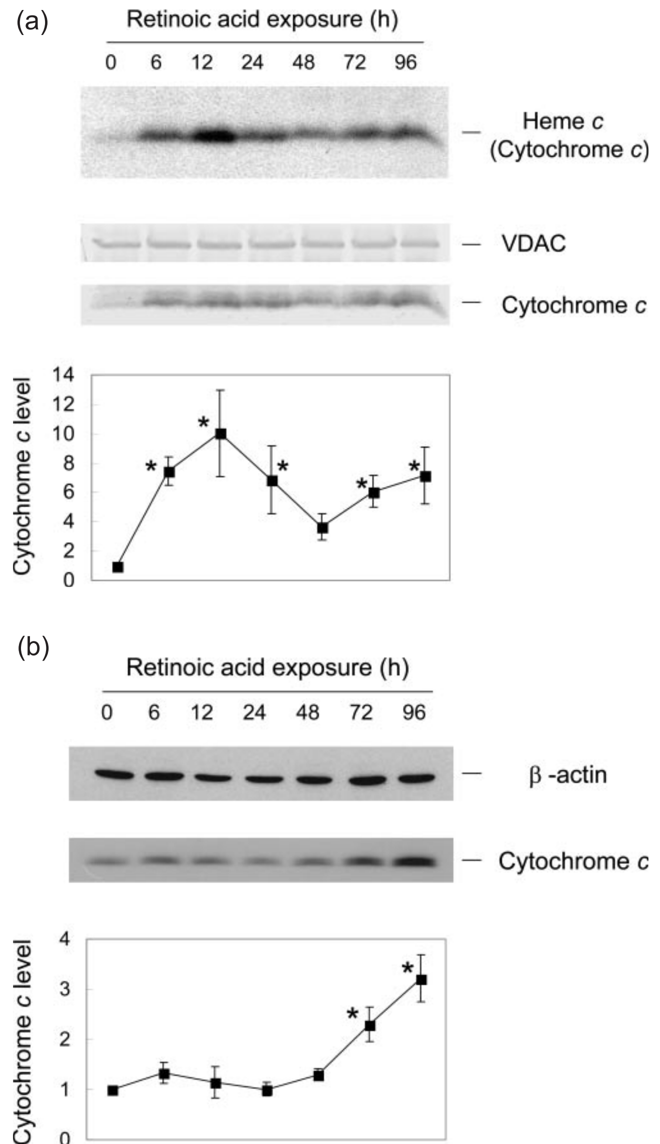

Fig. 5. **Changes in mitochondrial heme *c*, cellular cytochrome *c*, and cytochrome *c* mRNAs in differentiating Neuro2a cells.** (a) Mitochondrial proteins were separated by SDS-PAGE on a 10–20% gradient gel and then transferred to nitrocellulose filter. Heme *c* was detected by means of enhanced chemiluminescence (upper panel). Western blotting was performed with anti-voltage-dependent anion channel (VDAC; control protein) and anti–cytochrome *c* antibodies (middle two panels). Cytochrome *c* levels obtained on densitometric analysis (NIH Image 1.62) are also shown in comparison with the values at 0 h (lower panel). (b) Proteins in total cell lysates were separated by SDS-PAGE on a 15% gel, and cytochrome *c* (lower panel) and β-actin (upper panel) were detected by means of enhanced chemiluminescence using anti–cytochrome *c* and anti β-actin antibodies. The results for the mitochondrial and cellular cytochrome *c* levels represent means ± SD (n = 3). *$P < 0.05$ *vs.* 0 h.

cholinergic neuron-like differentiation of Neuro2a cells (*24*, *25*) were observed (*17*) after retinoic acid treatment (days 0–4). Neurites continued to grow during the period (days 0–4) and the activity of acetylcholine-esterase increased to day 3, but no further activation was observed from day 3 to day 4. We have cultured Neuro2a cells under the same conditions and studied them for 0–96 h after the onset of induction, which corresponds to "days 0–4" (*17*), because the differentiation of Neuro2a cells as cholinergic neuron-like cell occurred from start to 72 h, and neurite extension

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and maintenance of neuron-like cells continued to at least 96 h after the onset of induction.

Heme synthesis is known to be regulated during the differentiation of erythroid precursors. In these cells, the first step of the heme biosynthetic pathway, ALAS, is mediated by an erythroid-specific gene (ALAS-2). mRNA expression of ALAS-2 is highly up-regulated during erythroid differentiation to satisfy the cellular demands for heme. Nonerythroid cells, however, do not express ALAS-2. Instead, housekeeping ALAS (ALAS-1) functions as the first enzyme in the heme synthetic pathway. Like ALAS-2, the ALAS-1 level is regulated at the mRNA level: the stability of ALAS-1 mRNA is regulated by the free heme concentration (*26*, *27*), and its transcription is regulated according to the cellular demands for heme (*28*, *29*).

In this study, we found that the expression of ALAS-1 mRNA was up-regulated during neuronal differentiation, whereas the mRNA for ALAS-2 could not be detected. Because the ALAS-1 mRNA level is controlled by the cellular heme concentration (*26*, *27*), the increase in the ALAS-1 mRNA level may be related to enhanced mRNA stability due to the lower level of free heme (heme *b*) that was observed immediately after the induction of differentiation. In addition, the mRNA level for the downstream rate-limiting enzyme coproporphyrinogen oxidase was significantly increased, and a significant increase in protoporphyrinogen oxidase mRNA was also detected. The significance of the increases in the mRNA levels of these downstream enzymes is not clear, because there is little information about the regulation of these enzymes. However, since ALAS and coproporphyrinogen oxidase are rate-limiting enzymes for heme biosynthesis (*30*), it was supposed that activation of the heme synthetic pathway occurs during the differentiation of Neuro2a cells. Because the highest mRNA levels of several heme synthetic enzymes were observed around 48 h after differentiation induction, a high level of heme biosynthesis might be required around this time point.

Heme is an essential prosthetic group for many proteins, such as mitochondrial respiratory enzymes, globin proteins, and some oxidoreductases. Neuronal differentiation consumes a great deal of energy because of the many morphological and functional changes. Therefore, it is not surprising that heme biosynthesis should be enhanced to maintain energy production by the mitochondrial respiratory chain. However compared with the cellular heme *b* level, no significant increase in the mitochondrial heme *b* level was observed. The content of mitochondria per cell does not seem to increase during differentiation, because the only source of heme *a* is complex IV of the mitochondrial respiratory system, and both mitochondrial heme *a* and cellular heme *a* did not increase significantly. Therefore it was supposed that the increase in the cellular heme *b* level in the later phase of differentiation was not due to the triggering of mitochondrial respiration. An elevated heme *b* level may be needed for the production of neuron-specific heme proteins, including neuroglobin and neuronal nitric oxide synthase (*8*, *31*), or essential for expression of the key neuronal gene (*13*).

The most interesting observation in this study was the significant increase in heme *c* during the early phase of differentiation. The cytochrome *c* increase occurred prior to the increases in the mRNA levels of heme biosynthetic enzymes and at the same time as the decrease in heme *b*. In addition, succinyl-acetone, a heme biosynthesis inhibitor, did not interfere with the mitochondrial cytochrome *c* increase in the very early phase of differentiation (3 h after the onset of induction), although the decreases in the heme *a* and *b* levels were accelerated (data not shown). These results suggest that the increased localization of cytochrome *c* proteins in mitochondria was independent of the heme level, at least in the initial phase of differentiation. Cytochrome *c* is encoded in the nucleus, synthesized in the cytoplasm as apo-cytochrome *c* (lacking heme), and then translocated to the mitochondria. During or after mitochondrial import, heme is covalently attached *via* thioether linkages to two cysteine residues in the cytochrome *c* protein (*32*) and mature cytochrome *c* (holo-cytochrome *c*) is formed. The increased localization of cytochrome *c* proteins in mitochondria found in this study suggested that heme was preferentially consumed for the cytochrome *c* maturation in the early phase of differentiation. We also examined the level of cellular cytochrome *c* protein including apo- and holo-cytochrome *c* during differentiation. It was found that the cellular cytochrome *c* level did not change significantly during the early phase of differentiation, suggesting that the increase in cytochrome *c* is due to changes in post-translational steps, such as mitochondrial transport or heme attachment.

Because hemes *a* and *b* did not increase during this early period, the other components of the mitochondrial respiratory chain are also not expected to increase. Thus, it is unlikely that this increase in cytochrome *c* reflects activation of the respiratory chain. This leaves the question of what is the significance of this drastic increase in cytochrome *c*. Cytochrome *c* has many other roles besides acting as a component of the respiratory chain. For example, it is a scavenger of oxygen free radicals (*33*, *35*). Cytochrome *c* also interacts with the mitochondrial permeability transition pore (*36*, *37*), which forms the site of contact between the inner and outer mitochondrial membranes, and mediates the passage of metabolites between the mitochondria and the cytosol. This pore is known to participate in the release of apoptogenic factors during apoptosis, but its function as a calcium transporter in intracellular signaling has also been suggested (*38*). The physiological role and regulation of the mitochondrial cytochrome *c* increase should be elucidated, and the distribution of apo- and holo-cytochrome *c* in the early phase of nerve cell differentiation is now under investigation.

Neuronal differentiation is important not only for nerve development but also for nerve regeneration. Recently, it was shown that the central nervous system can regenerate due to the ability of precursor cells to differentiate into neurons and thereby compensate for damaged cells (*39*). This regenerating capacity should help protect the central nervous system from damage and stress related to aging. This could explain the reported correlation between the loss of heme and aging in the brain (*22*).

In this study, alterations of heme *a*, *b* and *c* metabolism, and the significance of heme biosynthesis during retinoic acid-induced neuronal differentiation of Neuro2a cells were suggested. This is the first report showing such changes, although other studies have demonstrated the

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importance of heme and the heme biosynthetic system in neuronal cells (*11–13*, *22*, *40*).

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