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Color reduction of melanin by lysosomal and peroxisomal enzymes isolated from mammalian cells

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Abstract Lysosomes and peroxisomes are organelles with many functions in all eukaryotic cells. Lysosomes contain hydrolytic enzymes (lysozyme) that degrade molecules, whereas peroxisomes contain enzymes such as catalase that convert hydrogen peroxide (H_2O_2) to water and oxygen and neutralize toxicity. In contrast, melanin is known as a helpful element to protect the skin against harmful ultraviolet rays. However, a high quantity of melanin leads to hyperpigmentation or skin cancer in human. New materials have already been discovered to inhibit tyrosinase in melanogenesis; however, melanin reduction does not suggest their preparation. In this study, we report that the color intensity because of melanin decreased by the cellular activation of lysosomes and peroxisomes. An increase in the superficial intensity of lysosome and peroxisome activities of HeLa cells was observed. In addition, a decrease in the amount of melanin has also been observed in mammalian cells without using any other chemical, showing that the process can work in vivo for treating melanin. Therefore, the results of this study indicate that the amount of melanin decreases by the lysosome and peroxisome activity after entering the cells, and functional organelles are effective in color reduction. This mechanism can be used in vivo for treating melanin.

Keywords Lysosomes · Peroxisomes · Melanin · Reactive oxygen species (ROS) · Degradation

Introduction

Lysosomes and peroxisomes are significant organelles in all eukaryotic cells [1]. Lysosomes, reported in the mid-fifties as cellular organelles full of acid hydrolases, play an important role in a number of biological processes in eukaryotes. Some special lysosomes also secrete cytotoxic molecules or melanin. The degradation of lysosomes generally takes place by endocytosis, providing extracellular material for digestion, whereas an autophagic process is implicated in the degradation of cytoplasmic constituents and is activated in response to the stress conditions [2]. Recently, lysosomes isolated from egg white have been reported to show pH-dependent antimicrobial activity with the optimum activity at pH 6.0 [3, 4, 5]. On the other hand, peroxisomes are the only organelles whose physiological role depends upon cell type, tissue, and metabolic state of the organism [6–8]. Peroxisomes participate in various metabolic pathways including beta-oxidation of very long chain fatty acids, biosynthesis of cholesterol, and detoxification of xenobiotic and reactive oxygen species (ROS). Approximately 20 peroxisomal disorders are associated with peroxisomal biogenesis disorders [9, 10].

Melanin is polymorphs and multifunctional biopolymers, and is the end product of the multistep transformations of L-tyrosine [11]. Melanin pigments impart color to the skin, eyes, and hair and consist of many units linked by carbon and carbon bonds, but differ in their chemical

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composition, structure, and properties. The two widely studied melanins are (1) eumelanins, which are nitrogenous biopolymers brown–black in color, insoluble in many solvents, and contain a high content of carboxyl groups and (2) pheomelanin, which is reddish-yellow in color and soluble in alkalis, has a backbone of benzothiazine units, and contains nitrogen and sulfur in its structure [12].

It can also act as a binding agent for drugs and chemicals. Pheomelanin is responsible for fair skin color, whereas the presence of abundant protective dark eumelanin imparts pigmentation in colored individuals. Although melanin acts as photosensitizers under UV light and generate ROS, superoxide anion, and H_2O_2, they also act as the scavengers of a number of oxidizing and reducing radicals. Melanin is associated with the development of melanoma; exposure to UVA radiation is a risk factor for melanoma [13]. Exposure to UVA (320–400 nm) radiation is associated with the generation of singlet molecular oxygen (1O2), a ROS that damages cells by oxidizing amino acids, lipids, and DNA. Dysplastic nevi, a phenotype that presents before melanoma development, has abnormal melanosomes, the organelles that synthesize melanin. The leakage of melanogenic intermediates and related enzymes out of melanosomes bring melanin in close proximity to DNA and lead to the possibility of DNA-melanin interactions. Thus, eumelanin can lead to DNA strand breakage even in the absence of radiation [14–16].

As mentioned above, melanin has harmful effect. Melanin reduction by lysosome has been reported in 1971 [2]. Focused lysosomal activity related to the melanin reduction in transfected fibroblast cells was reported in 1997 [17]. In our study, we tried to understand that lysosome used to reduce melanin amount using their function in the cells. In addition, the mechanism of ROS is related to enzyme activation was established, decreasing melanin amount.

Materials and methods

Cell culture and prepared melanin solution

HeLa cells (HC18802) were grown at 37 °C in a medium under 5 % CO_2, and cultured in Dulbecco's modified Eagle medium (DMEM) to which 5 % NCS (Newborn calf serum) and 5 % penicillin streptomycin were added. In each experiment, the medium of the cells was changed every 2 days. The eumelanin reagent was purchased from Sigma Chemical Co. (St. Louis, USA) and dissolved in 0.1 M sodium phosphate buffer (pH 7.0) to make melanin solution. After the HeLa cells were grown to 70 %, they were treated with this melanin solution [18].

Lysosomal and peroxisomal enzymes isolation

HeLa cells were rinsed twice with phosphate-buffered saline (PBS), treated with a NP-40 buffer added with Phenylmethylsulfonyl fluoride (PMSF) and Protease inhibitor cocktail (PIC). All the cells were racked out from the dishes with a scraper and placed in conical tubes. The cells were vortexed for 10 min and kept in ice for 30 min. Then the mixture was centrifuged at 3000 rpm for 5 min to remove the cell debris. The supernatant was transferred to microtubes and ultra-centrifuged at $20,000 \times g$ for 30 min to separate the lysosomes and peroxisomes from the supernatant. After discarding the supernatant, the lysosomes and peroxisomes were collected in the pellet form. The pellet was then mixed with NP-40 buffer in 1:1 ratio. The cells were vortexed for 15 min and kept in ice for 30 min. The mixture was centrifuged at $13,000 \times g$ for 10 min. The lysosomal and peroxisomal enzymes remained in the supernatant [3, 4, 18].

Tyrosinase inhibition activity assay

Tyrosinase is an important enzyme in the process of the preparation of melanin during melanogenesis [19–22]. For this experiment, the lysosomal and peroxisomal extracts were required. HeLa cells were cultured in two groups: one of them being normal grown cells and second, the cells were exposed to melanin for 12, 24, and 48 h. Lysosomal and peroxisomal enzymes were isolated by the enzymes' isolation method.

The tyrosinase activity was determined using L-DOPA (Sigma-Aldrich, St. Louis, MO, USA) as the substrate. Mushroom tyrosinase (500 unit/mL, Sigma, St. Louis, MO, USA) was prepared in 0.1 M sodium phosphate buffer (pH 6.8). All the reagents were mixed following this method in a 96-well plate. The composition of different samples are as follows: OD_{sample} containing prepared extracts (100 μL), tyrosinase (20 μL), and 1.5 mM L-DOPA (80 μL); OD_{blank} sample containing prepared extracts (100 μL), PBS (20 μL), and 1.5 mM L-DOPA (80 μL); $OD_{control1}$ sample containing 0.1 % NP-40 buffer (100 μL), tyrosinase (20 μL), and 1.5 mM L-DOPA (80 μL); and $OD_{control2}$ sample containing 0.1 % NP-40 buffer (100 μL), tyrosinase (20 μL), and 1.5 mM L-DOPA (80 μL). All these samples were incubated at 37 °C for 10 min, and their absorbance was measured at 450 nm by an ELISA reader [19, 24–26].

$$\text{Tyrosinase Inhibition Activity } (\%) = \left[1 - \frac{OD_{sample} - OD_{blank}}{OD_{control1} - OD_{control2}} \right] \times 100.$$

Melanin assay

Two groups of samples were prepared, and HeLa cells were cultured on five dishes exposed to melanin for 0, 12, 24, 48, and 72 h. The first group was treated with 100 ppm melanin solution, and the second group was cultured normally and used as the control. After reacting for 3 days, the cells were rinsed twice using phosphate-buffered saline (PBS) and further treated with 1× trypsin EDTA for tearing them off. Cultured cells on independent dishes were taken in 50-mL tubes and were centrifuged for 5 min at 3000 rpm. The supernatant was removed, and a part of the cells were taken in microtubes with 300 μL sterile water and sonicated five times in 20 % AMP for 10 s times one by one. In this step, melanin exited the cells if melanin reagent was present. Finally, melanin in the remaining supernatant was centrifuged at 13,000×g for 10 min.

The amount of melanin was calculated in comparison with the control 1 (C_1) for the cells exposed to 100 ppm melanin and incubated for cells that were incubated normally in 5 % CO_2 and at 37 °C. All the values were calculated by using the following formula.

Amount of melanin = $\text{Control}_1(C_1) - \text{Control}_2(C_2)$.

Fluorescence activity

The effect of the activity of cell organelles was also examined by Lyso-Tracker, and the effect of peroxisomes with peroxi-GFP Organelle Lights was also investigated. The cells were grown in a coverglass-Botton dish, rinsed, and stained with peroxi-GFP Organelle Lights kit (Molecular Probes, Leiden, The Netherlands) followed kit methods. Two types of reactive oxygen species (ROS) dye were used, one purchased from Cellrox (deep red reagent, Invitrogen, Carlabad, CA, USA) and the other from DCF-DA (Sigma, CA, USA).

In addition, the sample cultured cells were stained with 1 nM Lyso-Tracker Red DND 99 (Molecular Probes, Leiden, The Netherlands) in DPBS for 1 min 30 s at 37 °C. All the cells were mixed with 4 % paraformaldehyde for 30 min at room temperature. After 30 min, the supernatant was rinsed with PBS. All the samples were observed by laser-scanning microscopy (LSM 510 META), using the light of wavelength 543 nm for lysosomes and 488 nm for peroxisomes. Images were generated using Zeiss LSM image Browser [3, 4, 18].

Peroxidase activity assay

The peroxidase activity in this study was measured using the peroxidase activity kit (BioAssay, Haward, CA, USA). The peroxidase activity kit is made up of three reagents: calibrator, working, and stop reagent. The working reagent consisted of assay buffer, dye reagent, and 0.6 % H_2O_2. The lysosomal and peroxisomal proteins were extracted after HeLa cells were exposed to melanin for 12, 24, and 48 h. The blank was PBS (10 μL). Each 10 μL sample was mixed with 90 μL working reagent and reacted for 10 min at room temperature. Then, the stop reagent (100 μL) was added with sterile water and calibrator (200 μL), and all the samples were placed in a 96-well plate, and the activity was measured using an ELISA reader at 560 nm. All the values were calculated by using the following formula [27, 28].

$$\text{Peroxidase Activity (IU/L)} = \frac{\text{OD}_{\text{sample}} - \text{OD}_{\text{blank}}}{\text{OD}_{\text{calibator}} - \text{OD}_{\text{DW}}} \times 1000.$$

Data analysis

All the data were obtained from three independent samples, run simultaneously for error analysis, and the results are reported with the standard deviations. The data were analyzed using Sigma Plot (SPS, Chicago, IL, USA). A P value of <0.05 was considered significant.

Results and discussion

Before the activities of lysosomes and peroxisomes can be confirmed in cells, we need to know whether melanin, which is a huge molecule, has entered the cells or not [23]. After 100 ppm melanin solution was added to the cells for 0, 12, 24, 48, and 72 h, the quantity of melanin was measured using an ELISA reader at 450 nm (Table 1). The amount of melanin increased with time, and ∼18 ppm melanin was measured after 24 h (Table 1). This shows that melanin molecules enter the cells during the first 24 h. However, after 24 h, the amount of melanin showed a sharp decrease, reaching zero; and no melanin was detected at 48 and 72 h. Although not a part of our study, the amount of melanin after 24 h may be due to melanin saturation, or the cells might degrade the melanin components.

To evaluate the effect of lysosomes and peroxisomes, the activity was examined by Lyso-Tracker (for lysosomes) and peroxi-GFP Organelle Lights (for peroxisomes). The effect of the fluorescent intensity of lysosomes and peroxisomes on HeLa cells exposed to melanin was also studied as a function of time (Fig. 1). The fluorescent intensity increased with time for both lysosomes and peroxisomes, showing that lysosomal and peroxisomal enzymes responded to and were activated by melanin after the HeLa cells were exposed to the melanin environment for a long time. In addition, the average fluorescence intensity per unit surface area of the cells was determined (Fig. 2). Both the lysosomal and

Table 1 Amount of melanin (C_1–C_2) after exposure for different times

	Reaction times (h)				
	0	12	24	48	72
Amount on melanin (ppm)	4.070 ± 0.032	10.77 ± 0.314	18.38 ± 0.142	0	0

C_1, the amount of melanin for cells cultured with melanin for 0, 12, 24, 48, and 72 h; C_2, the amount of melanin for cells cultured normally without melanin for 0, 12, 24, 48, and 72 h. The melanin amount was measured after breaking the cells

All value calculated that C_1 (exposed melanin to cells for 12, 24, 48 and 72 h)—C_2 (cultured normally)
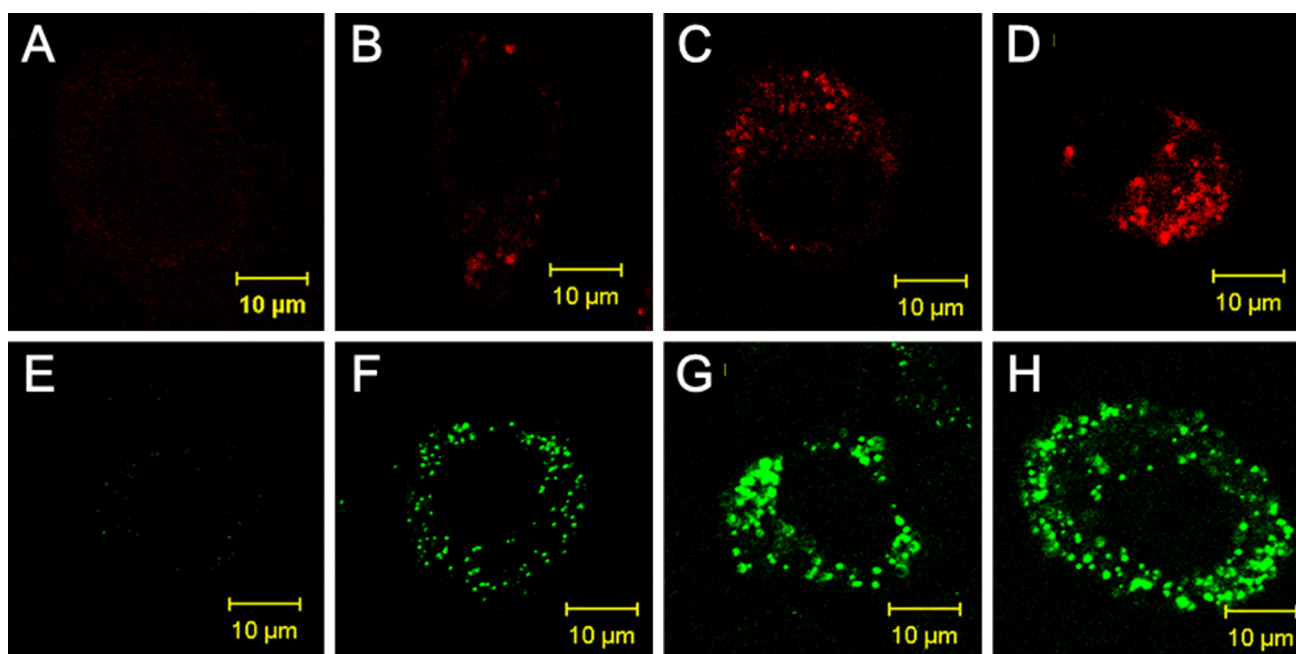
Fig. 1 Fluorescence of lysosomes (**a–d**) and peroxisomes (**e–h**) after organelles reacted to melanin for 0, 12, 24, and 48 h

peroxisomal activities showed an increase with exposure time of melanin. The increase in the lysosomal activity was exponential, whereas the peroxisomal activity showed a nearly linear increase. Following this result, because lysosomal and peroxisomal enzymes are activated by melanin, the activities of the enzymes isolated from mammalian cells need to be confirmed.

In contrast, tyrosinase is a key enzyme of melanogenesis, related to tyrosine, L-Dopa (L-3,4 dihydroxyphenylalanine), and dopaquinone [19–22]. The tyrosinase inhibition activity (TIA) by lysosomal and peroxisomal extracts was studied, as these extracts were expected to have an effect on melanogenesis. For this experiment, all the extracts isolated from HeLa cells were exposed to melanin for 0, 12, 24, and 48 h. After the extracts were treated with L-DOPA and tyrosinase, the TIA was measured, as shown in Fig. 3. The TIA decreased with time. This result proves that the lysosomal and peroxisomal extracts are not related to the inhibition of tyrosinase enzyme on melanogenesis.

Oxidative stress is well known to occur as the concentration of ROS increases, and the high levels of ROS have a toxic effect on various biomolecules such as DNA, lipids, and proteins. When the cells are exposed to chemicals, they suffer from oxidative stress as ROS are constantly generated and eliminated by various biological mechanisms [9, 10]. By measuring the ROS activity and lysosomal activity by confocal microscopy, any change in ROS because of its treatment with melanin can be determined. The fluorescence activity of lysosomes and ROS is shown in Fig. 4a–l, indicating that there are ROS spreads in cytoplasm, and the activities of the lysosomes slightly merged. The cells exposed to melanin for 48 h shows more merging of the fluorescence of lysosomes with ROS, indicating that lysosomes are related to the reduction of oxidative stress, generated from melanin. In contrast, Fig. 4m–x shows the fluorescence activity of peroxisomes and ROS as a function of time. In this case, peroxisomes are not affected by the treatment with melanin. Although the peroxisomal activity increased with time, ROS did not merge with peroxisomes.
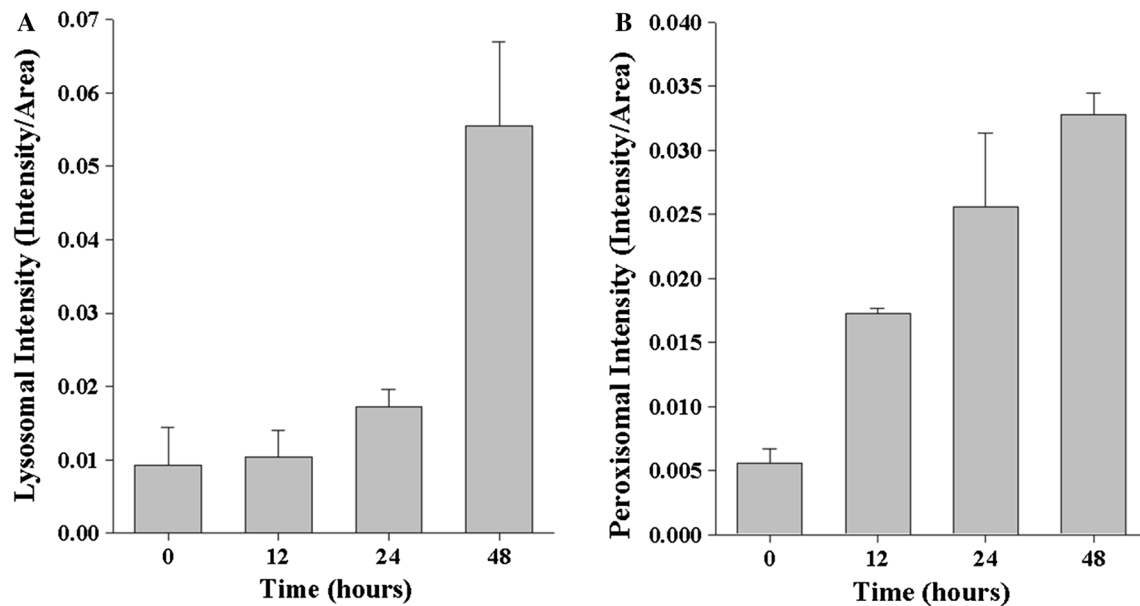
Fig. 2 *Bar diagram* showing the average fluorescence intensity of activities (intensity/area) for lysosomal (**a**) and peroxisomal (**b**) after organelles reacted to melanin for 0, 12, 24, and 48 h
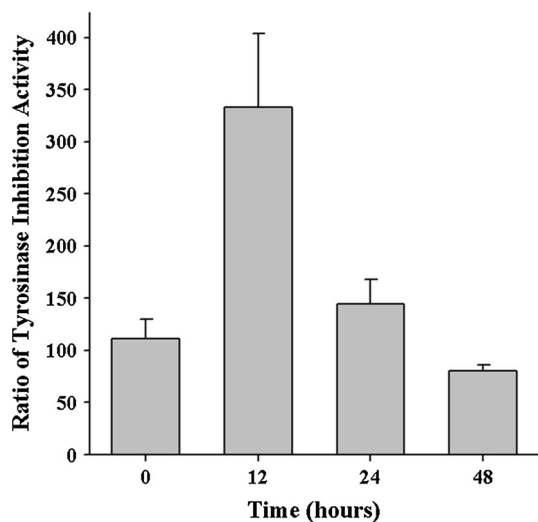
Fig. 3 Tyrosinase inhibition activities (TIA) of lysosomal and peroxisomal extracts isolated from the HeLa cells exposed to melanin for 0, 12, 24, and 48 h

If the lysosomal and peroxisomal enzymes decreased the amount of melanin and color, then the effective enzyme, which may be responsible for this activation should be investigated. Peroxidase is typically an important enzyme in peroxisomes [9, 10]. Lysosomal and peroxisomal enzyme from the HeLa cells exposed to melanin was extracted to confirm the peroxidase activity. Figure 5 shows the peroxidase activity as a function of time. The peroxidase activity increases similar to that observed for the peroxisomal activity (Fig. 2b). From these results, we

propose that peroxisomes are probably related to the decreasing amount of melanin in the present case. However, further study is required to explain this behavior.

Lysosomes and peroxisomes are organelles in eukaryotes with antimicrobial and anti-cancerous activities. In this study, melanin reduction by lysosomal and peroxisomal enzymes was investigated. The level of melanin depends upon race, amount of exposure to sunlight, and hormonal changes. However, large amounts of melanin can be harmful. Usually sun-exposure promotes the melanin synthesis in epidermis by activating various enzymes. For instance, a high quantity of melanin overexpresses EDNRB, leading to the loss of C-kit expression observed in human melanoma [13, 14]. This process may cause erythema (sunburn inflammation), immune-suppression, DNA damage, and cancer. Furthermore, a moderate concentration of melanin is required for healthy skin, though melanin poses harmful effect to human.

Lysosomes and peroxisomes were activated by melanin, and the fluorescence intensity of lysosomes and peroxisomes in the HeLa cells exposed to melanin increased with time. The peroxidase activity measured by using the extracts isolated from the HeLa cells exposed to melanin showed that enzymes were activated by melanin. The extracts of lysosomal and peroxisomal were also treated with L-Dopa (L-3,4-dihydroxyphenylalanine) to measure the TIA. Lysosomal and peroxisomal extracts are not effective in inhibiting enzymes in the melanogenesis mechanism. The oxidative stress of the HeLa cells increased after the treatment with melanin, and the results for lysosomes and peroxisomes with
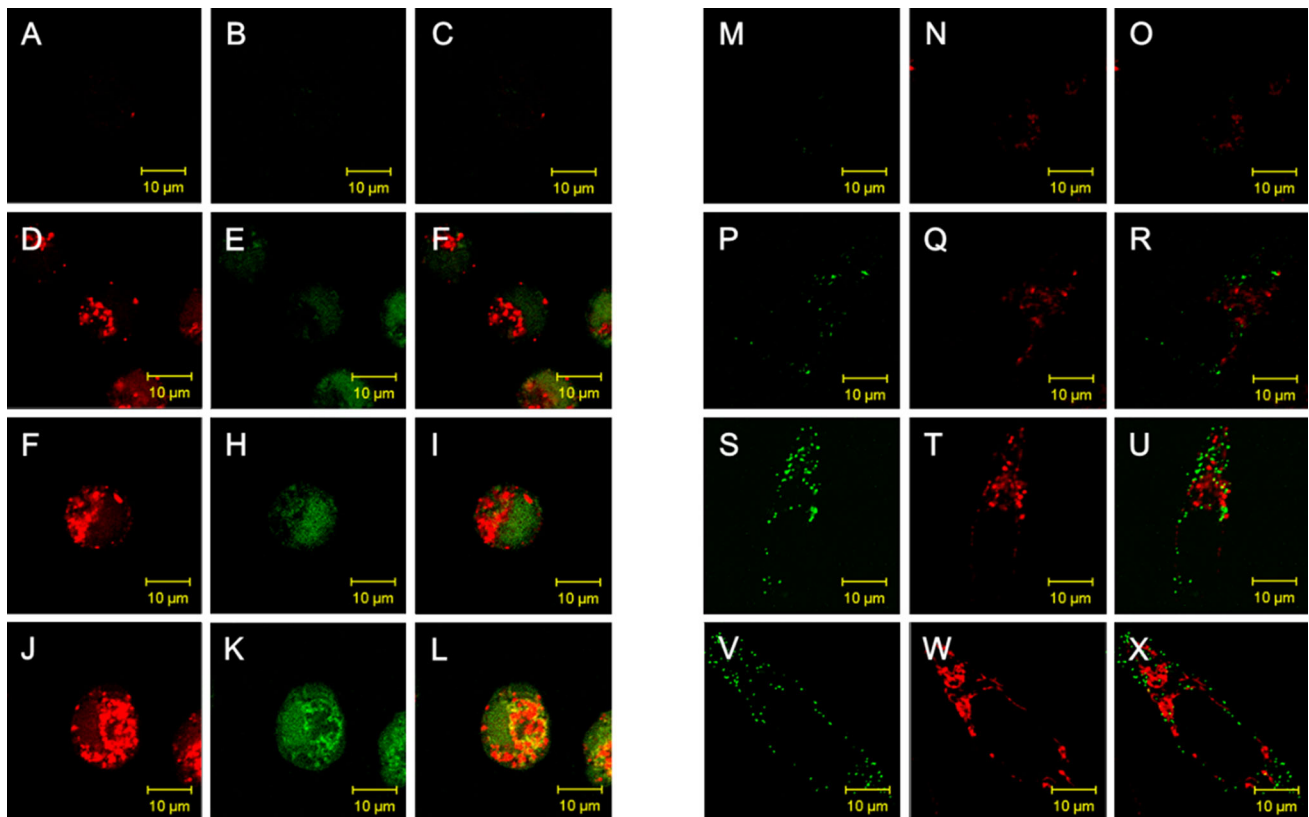
Fig. 4 Fluorescence of lysosomes and ROS exposed to melanin for 0 (*a–c*), 12 (*d–f*), 24 (*g–i*), and 48 (*j–l*) h, and fluorescence of peroxisomes and ROS exposed to melanin for 0 (*m–o*), 12 (*p–r*), 24 (*s–u*), and 48 (*v–x*) h
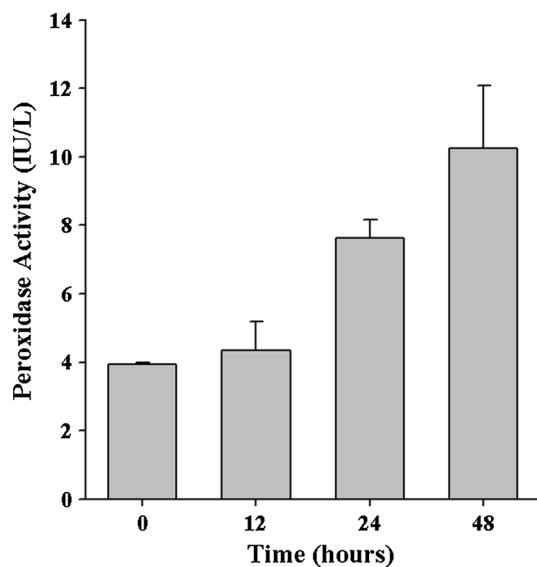
Fig. 5 Peroxidase activity of the enzymes isolated from lysosomes and peroxisomes on HeLa cells at different times

ROS were compared. With an increase in the oxidative stress, lysosomal activity shows more effect than peroxisomes. Lysosomes are generally assumed to be more sensitive to the oxidative stress caused by melanin.

The quantity of melanin decreases by the cellular activation of lysosomes and peroxisomes. Thus, the enzymes isolated from this functional organelle in mammalian cells are potential new materials and can be used to decrease melanin.

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