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Original Research Article

Luminol-Enhanced Chemiluminescent Response of Human Melanocytes and Melanoma Cells to Hydrogen Peroxide Stress

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The response of human melanocytes and melanoma cells to hydrogen peroxide stress was measured. Cells were exposed to glucose/glucose oxidase or free H₂O₂ and reactive oxygen species measured by luminol-enhanced chemiluminescence. The response was distinctly different between the two types and the addition of superoxide dismutase to melanoma cells paradoxically enhanced the chemiluminescent signal. These findings coupled with other known differences between the way these two types of cells handle oxidative stress at a molecular level suggests that a therapeutic window may be available for exploitation.

Key words: Melanocytes, Melanoma, Reactive Oxygen Species

INTRODUCTION

Cutaneous human melanocytes function to produce melanin in response to ultraviolet light (UVL). Increasing evidence indicates that changes in the redox state are affected by UVL and that H₂O₂ serves as an important mediator of downstream events, including activation of various signaling pathways and transcriptional factors (Bertling et al., 1996; Pacher and Sen, 1996; Suzuki et al., 1997). Melanoma cells have low catalase activity and glutathione levels compared to other cells (Picardo et al., 1996) and therefore appear to be in state of chronic oxidative stress, probably as a result of the endogenous generation of reactive oxygen species (ROS) as a byproduct of melanin synthesis. The situation is complex however since melanin itself may serve as a pro- or anti-oxidant depending upon the underlying redox state (Prota, 1992).

These observations suggest that melanocytes and melanoma cells may have a differential capacity to handle ROS. We have therefore begun to assess the management of oxidative stress by normal and malignant human melanocytes (Meyskens et al., 1996). In the current report we have examined the response of human melanocytes and melanoma cells to H₂O₂ using a glucose/glucose oxidase generation system or free H₂O₂ and luminol-enhanced chemiluminescence as the detection method (Fig. 1).

MATERIAL AND METHODS

Cell Culture

Human melanocytes were cultivated in MCDB 153 medium (Sigma Chemical Co., St. Louis, MO) with various growth additives as described by Yamanishi et al. (1991a,b).

Pooled neonatal foreskins (3-5 per cell line) were used to derive the melanocyte cell lines. Human metastatic melanoma cell strains were cultured in Ham's F-10 medium supplemented with 5% fetal calf serum, 5% newborn calf serum, penicillin (100 U/ml), and streptomycin (0.1 mg/ml) and have been previously described (Yamanishi et al., 1991a,b). The human laryngeal squamous cell carcinoma line (cSCC-20) and the human lung carcinoma line (Lu-CSF-1) were cultured in RPMI medium (Sigma Chemical Co., St. Louis, MO) with the same additives as the melanoma cell strains and have previously been described (Sun et al., 1990; Redpath et al., 1995). All medium contained 1.081 g/L of D-glucose. Cell viability was determined by trypan blue (0.4%) exclusion.

Chemicals

Hydrogen peroxide 30%, luminol, lucigenin, sodium hydroxide, and natural melanin were from Sigma Chemical Co. (St. Louis, MO). Glucose oxidase, superoxide dismutase and catalase were from Boehringer Mannheim (Indianapolis, IN). All chemicals were kept on ice until addition.

Chemiluminescence Assay

Cells were seeded at a density of 10⁴ cells/well into a 96 well clear bottom luminescent plate (Costar) and in-

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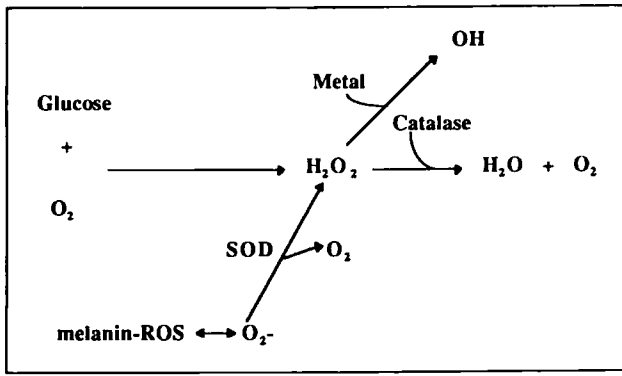


Fig. 1. Generations of hydrogen peroxide by glucose/glucose oxidase.

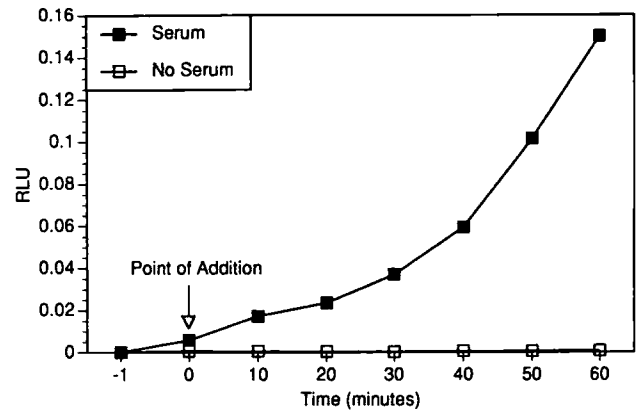


Fig. 2. Effect of serum on luminol-enhanced chemiluminescence without cells, square, serum; square, no serum.

cubated for 24 hr. Media was removed and cells washed in serumless media and replenished with serumless media. Luminol (5 μ M) or lucigenin (100 μ M) was added to each well in low light environment. Chemiluminescence was analyzed by luminometer (Dynatech Laboratories, Chantilly, VA) at 37°C. All assays were done in serum-free media as substances in serum produce an artifactual CL signal with luminol as the probe (Fig. 2); no signal was detected (even at maximum gain) in the absence of serum and cells.

Melanin Assay

Cells were harvested and washed in 1x PBS and the pellets were frozen at -80°C overnight. Cell pellets were resuspended in freshly made 1 M NaOH and allowed to shake at room temperature for 2 hr. Optical density was measured at 475 nm and standardized with natural melanin.

RESULTS

Effect of Glucose/Glucose Oxidase Generated Hydrogen Peroxide on Luminol-enhanced Chemiluminescence in Melanocytes, Melanoma, and Other Cell Types

Glucose/glucose oxidase generated hydrogen peroxide produced different CL responses in the cell lines measured (Fig. 3). No signal was detected in the presence of human keratinocytes or lung or head and neck carcinomas. A CL response in the melanocytes and melanoma cells was detectable but the kinetics between the two was strikingly different. In melanocytes a CL signal was noted almost immediately that peaked within 5–10 minutes and returned to baseline after about 30–45 min. In contrast all three melanoma cells responded slowly and the peak CL did not occur until after 50–60 min. The CL peak in melanoma cells ranged from 5–10 times that detected in the melanocytes and was still well above baseline even after 120 min. No

Luminol-Enhanced Chemiluminescence in Basal Melanocytes, Melanoma and other Cell Types

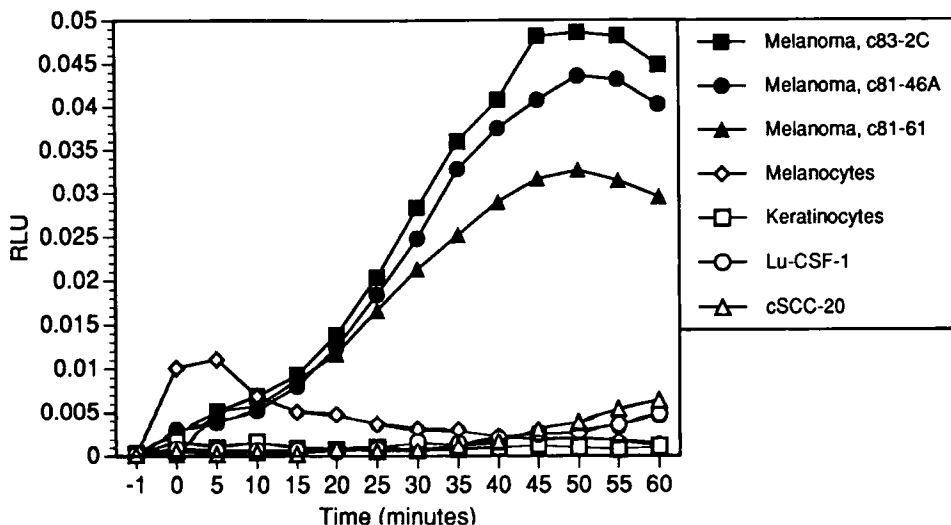


Fig. 3. Basal luminol enhanced chemiluminescence in melanocytes, melanoma, and other cell types. Closed square, melanoma 83-2C; closed circle, melanoma C81-46 A; closed triangle, melanoma C81-61; open diamond, melanocytes; open square, keratinocytes; open circle, lung carcinoma; and open triangle, oral carcinoma.

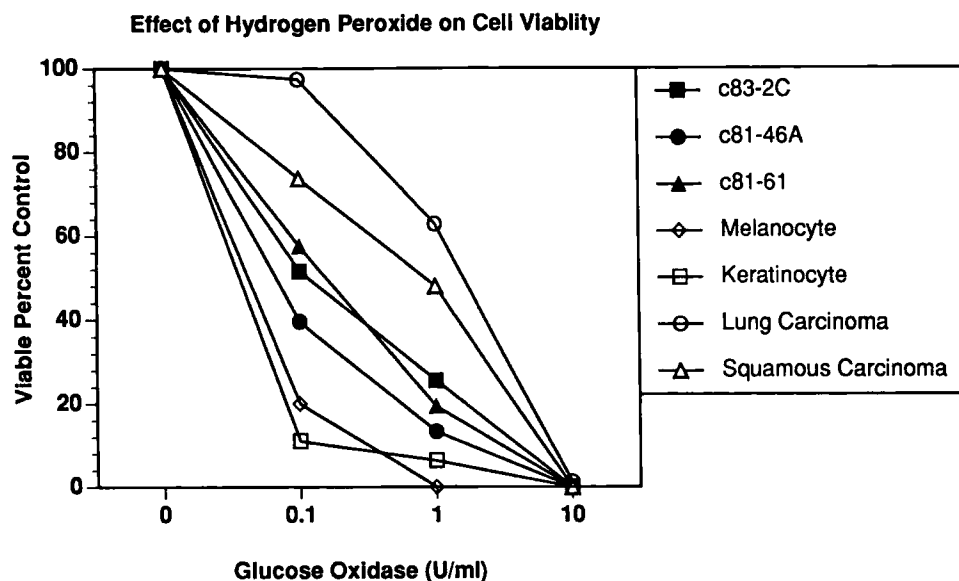


Fig. 4. Effect of glucose/glucose oxidase generated H_2O_2 on cell viability. Symbols as for Figure 3.

detectable signal was produced by either melanocytes or melanoma cells when lucigenin was used as the detector probe (data not shown).

Effect of Glucose/Glucose Oxidase Generated H_2O_2 on Cell Viability

Cells were treated with increasing doses of glucose oxidase in plain medium for one hour and cell viability measured by trypan blue exclusion (Fig. 4). The normal cells (melanocytes and keratinocyte) were similarly affected (LD_{25} , 0.1 glucose oxidase) and were considerably more sensitive than their transformed counterparts. The response of the melanoma cells from all three cell lines was quite similar (LD_{50} , 0.1 glucose oxidase) and considerably different than the two carcinomas (LD_{50} , >1.0 glucose oxidase). These results indicate that the killing effect of H_2O_2 can not be entirely explained by ROS, at least as measured by luminol-enhanced CL.

Effect of Catalase and Superoxide Dismutase on Luminol-Enhanced Chemiluminescence in Melanocytes and Melanoma Cells

Catalase was added to the medium containing melanocytes (after 5 min) or melanoma cells (after 35 min). As expected the CL-signal in both melanocytes and melanoma cells was promptly lost and suggested that H_2O_2 was actively contributing to the CL response. In contrast the addition of SOD produced strikingly different CL responses between melanocytes and melanoma cells. In melanocytes a slight decrease in CL signal was immediately evident but thereafter the kinetics were similar to the SOD untreated cells. In contrast the addition of SOD to melanoma cells produced an immediate and sustained increase in CL that was over five times that of untreated cells 30 min later. These results suggest that handling of superoxide anion ($O_2^{\cdot-}$), the major substrate for SOD, is different by melanocytes and melanoma cells.

Effect of Direct Addition of Hydrogen Peroxide on Luminol-enhanced Chemiluminescence in Melanocytes and Melanoma Cells

Hydrogen peroxide was directly added to cultures of melanocytes or melanoma cells (Figure 6). No chemiluminescent signal was generated by melanocytes until a concentration of H_2O_2 of 0.1% was reached. In contrast a CL signal could be detected in melanoma cells at a concentration as low as 0.001% H_2O_2 . These results suggest either that melanocytes are more effective at quenching ROS or that fewer ROS are generated by melanocytes

Effect of Glucose/Glucose Oxidase Generated H_2O_2 on Luminol-Enhanced Chemiluminescence in Melanocytes of Caucasian and African American Origin

The effect of H_2O_2 on luminol-enhanced chemiluminescence in cultured melanocytes from Caucasian and African Americans foreskins is shown in Figure 7. The CL response in African Americans derived melanocytes was 5–15% that of Caucasian derived melanocytes, suggesting a more efficient suppression of ROS.

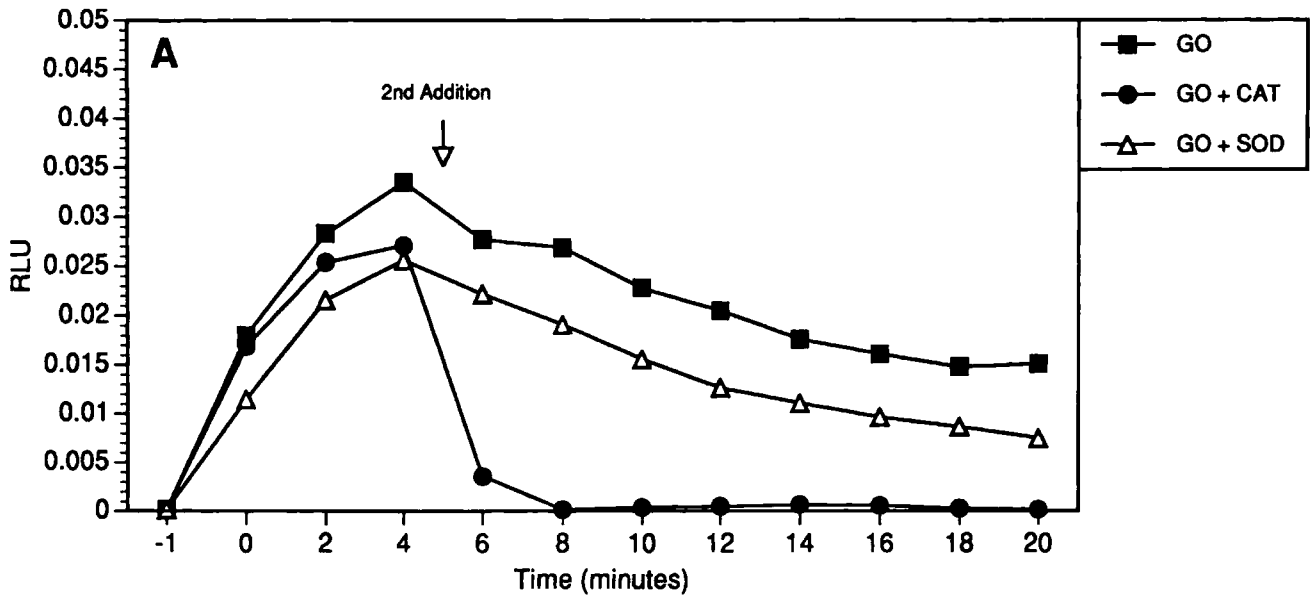
Measurement of Melanin Levels in Melanocytes and Melanoma Cells

The melanin content ranged from 10 to 15 units in the three melanoma cells and was 15 and 20 units in Caucasian and African American melanocytes, respectively.

DISCUSSION

These results suggest that melanocytes and melanoma cells respond to and handle oxidative stress differently. A comparison of the CL response in the melanocytes and melanoma cells exposed to H_2O_2 directly (Fig. 6) versus via glucose oxidase generation (Fig. 3) indicates that both cell types respond to H_2O_2 in a similar fashion but that melanocytes do so at a lower concentration of H_2O_2 , perhaps explaining in part the delayed response of the melanoma

Effect of Catalase and Superoxide Dismutase on Luminol-Enhanced Chemiluminescence in Melanocytes



Effect of Catalase and Superoxide Dismutase on Luminol-Enhanced Chemiluminescence in Melanoma Cells

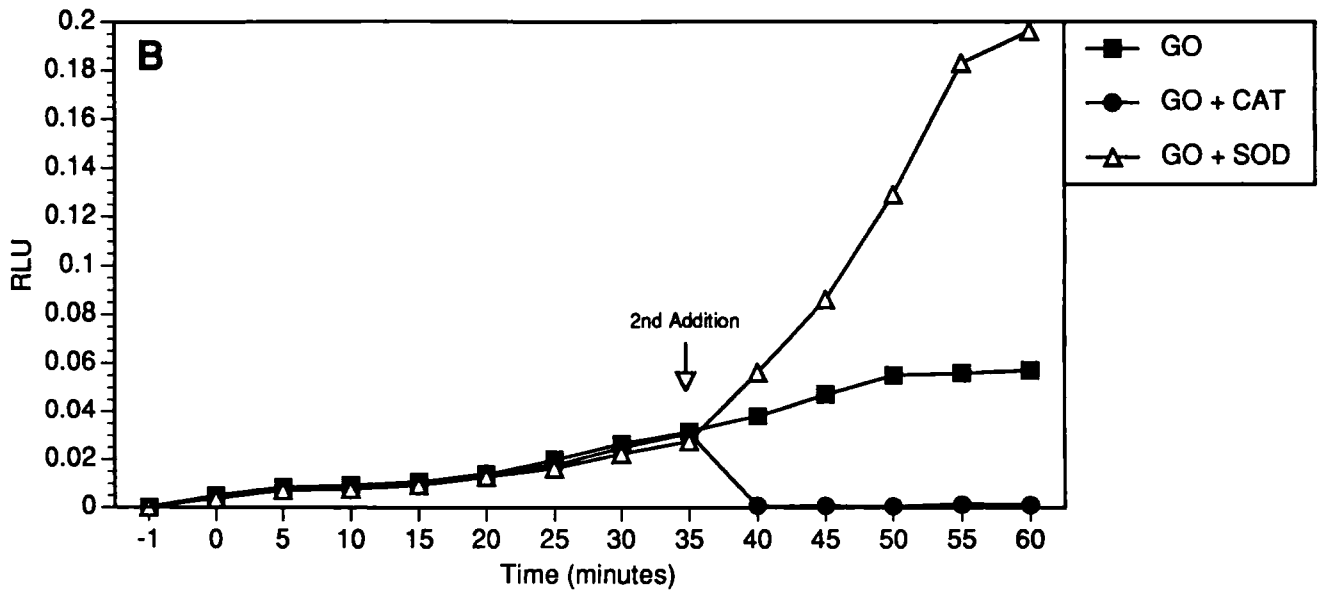


Fig. 5. Effect of glucose/glucose superoxide dismutase on luminol-enhanced chemiluminescence. melanocytes (A) and melanoma cells (B).

Closed square, glucose oxidase; closed circle, glucose oxidase plus catalase, and open triangle, glucose oxidase plus SOD.

cells to H₂O₂ generated by glucose/glucose oxidase in which H₂O₂ gradually accumulates. The higher CL signal in melanoma cells suggest that these cells are less able to handle oxidative stress compared to their normal counterparts. This is likely not to be related to melanin content alone as the total measured melanin content in Caucasian melanocytes is only 50% greater than that in the melanoma cells

tested. However, sequestration of ROS by melanin may be playing some role as ROS are clearly more suppressed in melanocytes derived from African American than from Caucasian foreskins (melanin content 20 vs. 15 units). Since UVL represents a contributory carcinogen to melanocyte transformation and melanoma incidence is known to be lower in dark skinned individuals, the nearly complete sup-

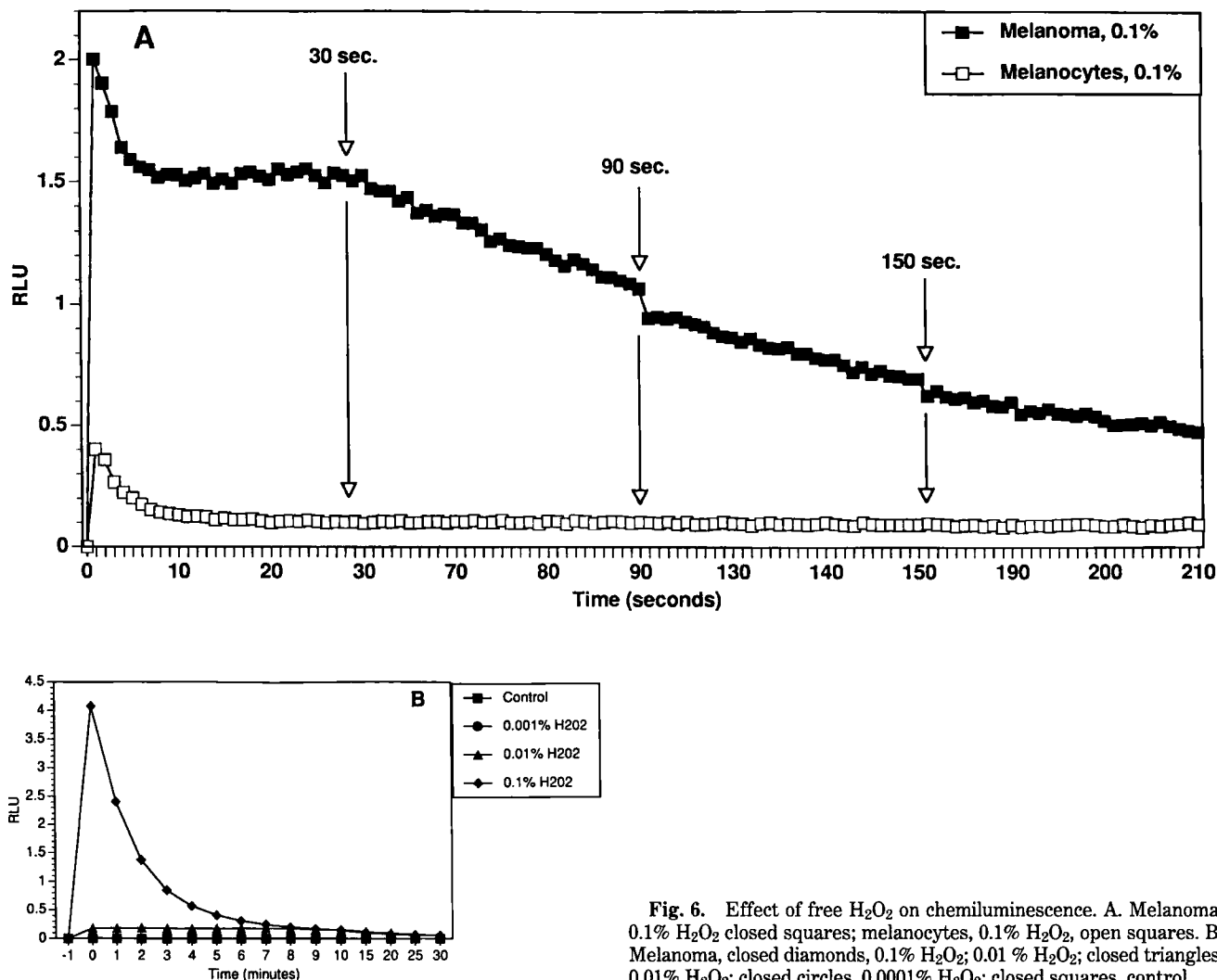


Fig. 6. Effect of free H₂O₂ on chemiluminescence. A. Melanoma, 0.1% H₂O₂ closed squares; melanocytes, 0.1% H₂O₂, open squares. B. Melanoma, closed diamonds, 0.1% H₂O₂; 0.01% H₂O₂; closed triangles, 0.01% H₂O₂; closed circles, 0.0001% H₂O₂; closed squares, control.

pression of ROS in melanocytes by melanin may be an important natural defense mechanism against oxidative stress induced damage.

The single most striking observation in the current study is the difference of CL response of melanocytes and melanoma cells after the addition of SOD. Since H₂O₂ is the major product of glucose and glucose oxidase, at least two phenomena must be simultaneously occurring to explain the divergent effect on CL between melanocytes and melanoma cells (Fig. 5). The small decrease in CL seen in the melanocytes after SOD is added suggests that a small superoxide anion pool is being depleted. However studies using lucigenin which selectively measures extracellular (e.g., membrane) O₂⁻ were negative for a CL response.

The marked increase of CL and hence ROS in melanoma cells in response to the addition of SOD is not easily explained without enlisting an immediate and active role for melanin that is different in melanoma cells compared to melanocytes. Melanin is a complex molecule that can function as either a pro-oxidant or anti-oxidant (Prota, 1992). A situation in which

melanin sequesters O₂⁻ or acts to donate electrons in the presence of high concentration of SOD would be the simplest of explanations, but determining the exact mechanism will be challenging.

In toto our results suggest that melanocytes and melanoma cells handle oxidative stress in a fundamentally different way. Others have shown that melanoma cells contain low level of catalase, SOD, and glutathione (Picardo et al., 1996). We have also recently shown that basal level and regulation of redox-sensitive transcription factors (Schmidt et al., 1995) in melanocytes and melanoma cells are markedly different as well (unpublished data). These observations and our data here suggest that melanoma cells have an altered and decreased redox capacity that may be amenable to experimental manipulation (Kang et al., 1993).

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**Effect of Hydrogen Peroxide on Melanocytes from
Caucasian and African Americans**

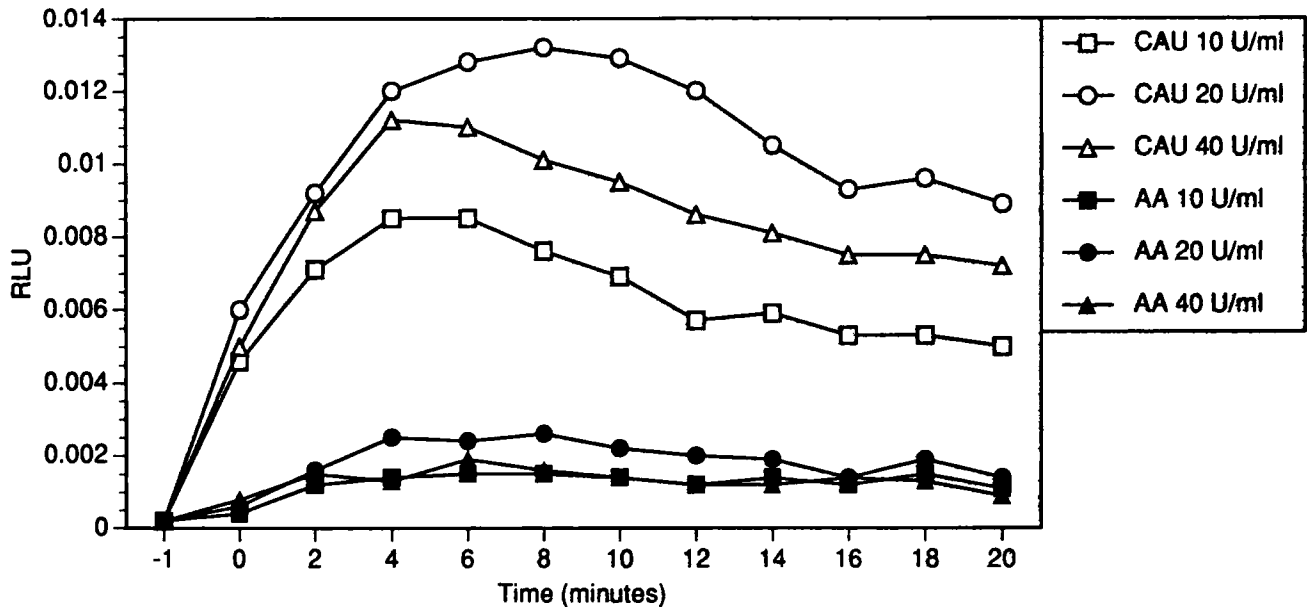


Fig. 7. Effect of glucose/glucose oxidase generated H_2O_2 on melanocytes of Caucasian and African American Origin. Symbols: open,

Caucasian; closed, African American. Glucose oxidase concentration: boxes 10 units/ml, circles 20 units/ml, and diamonds 40 units/ml.

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