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Glutathione peroxidase-1 inhibits UVA induced AP-2 α expression in human keratinocytes

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Abstract

In this study, we found a role for H₂O₂ in UVA induced AP-2 α expression in the HaCaT human keratinocyte cell line. UVA irradiation not only increased AP-2 α , but also caused accumulation of H₂O₂ in the cell culture media, and H₂O₂ by itself could induce the expression of AP-2 α . By catalyzing the removal of H₂O₂ from cells through over-expression of GPx-1, induction of AP-2 α expression by UVA was abolished. Induction of transcription factor AP-2 α by UVA had been previously shown to be mediated through the second messenger ceramide. We found that not only UVA irradiation, but also H₂O₂ by itself caused increases of ceramide in HaCaT cells, and C2-ceramide added to cells induced the AP-2 α signaling pathway. Finally, forced expression of GPx-1 eliminated UVA induced ceramide accumulation as well as AP-2 α expression. Taken together, these findings suggest that GPx-1 inhibits UVA induced AP-2 α expression by suppressing the accumulation of H₂O₂.

Keywords

Antioxidant enzyme; reactive oxygen species; signal transduction; skin; oxidative stress

Introduction

The solar ultraviolet (UV) spectrum can be divided into three wavelength ranges, UVA (315–400 nm), UVB (280–315 nm) and UVC (100–280 nm) [1]. Although there is evidence that UVA has the ability to induce pyrimidine dimers [2], the mechanism by which UVA causes biological damage is believed to be in the presence of oxygen and endogenous photosensitizers that produce reactive oxygen species (ROS) [3]. The primary type of ROS generated directly by UVA radiation is singlet oxygen (¹O₂) [4–6]. Nevertheless, other ROS can also be produced directly or indirectly as a result of UVA mediated cellular damage including superoxide anion, hydroxyl radical, hydrogen peroxide and other intermediates. These ROS collectively cause cellular oxidative stress, damage to cellular components [7], and genomic instability [8]. At low doses of UVA exposure, changes in signal transduction are predominant over oxidative

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stress [9]. Multiple signaling pathways have been shown to be induced by UVA including, p38 MAPK [10], AP-1 [11], AP-2 α [12] and c-MYC[13], and MMP1 [14].

It has been speculated that singlet oxygen may not be the sole or final ROS to cause the biological effects of UVA exposure. Hydrogen peroxide (H₂O₂) has been shown to be responsible for UVA induced DNA damage [15], and the inactivation of tyrosine kinase-regulating phosphatases by H₂O₂ and UVA are very similar [16]. Moreover, the lifetime of singlet oxygen in aqueous solution is about only 4 μ s [17] while H₂O₂ is relatively stable under physiological conditions [18].

Here we show that the UVA induced upregulation of the transcription factor AP-2 is due at least in part to the UVA induced increase in cellular H₂O₂. Over expression of the antioxidant enzyme GPx-1 attenuated the UVA induced upregulation of AP-2. Thus, we provide direct evidence for the hypothesis that H₂O₂ is a key ROS after UVA irradiation that stimulates the upregulation of AP-2 α expression in human keratinocytes.

Materials and methods

Cell culture

The human immortalized keratinocyte cell line HaCaT [19] was cultured at 37 °C, 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (Hyclone, Utah), 100 U per ml penicillin and 100 μ g per ml streptomycin.

UVA irradiation

The medium in 6-cm culture dishes was changed to serum free DMEM and incubated overnight before UVA irradiation. The cells were then washed with phosphate-buffered saline (PBS) and kept in 1 ml PBS during the subsequent irradiation. Cells were irradiated with a bank of four F20T12/BL/HO fluorescent UVA bulbs (National Biological Corp., Twinsburg, OH) with a peak emission at 365 nm. Radiation doses were measured using a UVX radiometer equipped with a UVX-36 sensor (UVP, Inc., Upland, CA).

H₂O₂ treatment

Before H₂O₂ treatment, HaCaT cells were cultured in serum free DMEM over night. The H₂O₂ was diluted from 30% stock solution (Fisher Scientific) to the indicated final concentration. The cells were incubated with H₂O₂ for 30 minutes in 1 $\times$ PBS and then cultured in fresh serum free DMEM for the indicated times.

Nuclear protein extraction

The nuclear proteins were extracted as previously described [20].

Western blot

Fourty μ g nuclear proteins from each group were loaded into each well for western blot to detect AP-2 α . The western protocol was previously described [20]. GAPDH or β -actin was used as loading control. The antibodies and titer used were: Rabbit Anti-AP-2 α 1:500 (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-GAPDH, 1:10,000 (Ambion, Austin, TX); rabbit anti-Actin, 1:2,000 (Sigma, St. Louis, MO) and rabbit anti-GPx-1, 1:2,000 (LabFrontier, Seoul, Korea).

Quantitative RT-PCR

Total cellular RNA was extracted from HaCaT with RNeasy® Mini Kit (Qiagen, Valencia, CA) at the indicated times after treatment. Two μ g of total RNA was reverse transcribed to

cDNA with High Capacity cDNA Archive Kit (ABI, Foster City, CA). Quantitative real time PCR was performed with ABI TaqMan® master mix and primer and probe assay. For AP-2 α , the ABI assay ID was Hs00231461_m1. β -actin was used as a control for RNA input and integrity, and its ABI assay ID was Hu ACTB. The PCR reactions were set up according to the manufacturers' instructions. The total reaction volume was 25 μ l. The signals were detected and analyzed with an ABI 7000 sequence detection system.

Reporter gene constructs

An AP-2 responsive chloramphenicol acetyltransferase (CAT) reporter that contains 12 AP-2 binding sites upstream of the CAT coding sequence was used as an AP-2 specific promoter-reporter. This reporter was used to detect the AP-2 transcriptional activity. Each 60 mm plate of cells was co-transfected with 5 μ g of CAT reporter plasmid and 5 μ g β -gal plasmid. After treatment, total RNA was extracted from the cells after 18 h. The reporter activities were expressed as relative CAT mRNA level/ β -gal mRNA levels. The primer sequences for CAT were: forward: 5'- AAT GAA AGA CGG TGA GCT GGT G; reverse: 5'- ACA CGCCAC ATC TTG CGA A. For β -gal: forward: 5'-ACA CCA GCA GCA GTT TTT CCA G; reverse: 5'-TCC AGT GCA GGA GCT CGT TAT C.

Overexpression of GPx-1

The GPx-1 was delivered by recombinant adenovirus as previously described [21]. Briefly, HaCaT cells were infected with 100 MOI Bgl II (control) or Ad-GPx-1. Western blots [22] and GPx-1 activity assays [23] were performed to confirm the overexpression of GPx-1 as previously described.

Measurement of exogenous H₂O₂

The H₂O₂ concentration changes upon UVA irradiation were measured by the pHRP method as previously described [24]. To ensure the signals were specifically from H₂O₂, either native or boiled catalase protein (5000 U) (Sigma, St. Louis, MO) was added to the buffer.

Intracellular peroxide measurement

The intracellular peroxide concentration was measured with 2', 7' - dichlorodihydrofluorescein diacetate (DCFH-DA) as previously described [25]. Briefly, cells were washed with 1 $\times$ PBS, and 3 ml of 10 μ M DCFH-DA (20 μ l of 20 mM stock in 40 ml PBS, Molecular Probes) was added to each plate and incubated at 37 °C for 30 minutes. Cells were washed with PBS, and then lysed with 800 μ l of 0.5% NP-40. Cell lysates were centrifuged at 10,000 $\times$ g at 4 °C for 3 minutes. 180 μ l from each sample was loaded into 96-well NUNC® black bottom plate and read in a SPECTRAFLUOR Plus microplate reader (TECAN, Austria) with excitation wavelength of 485 nm and emission wavelength of 530 nm. The protein concentration in the remaining lysate was determined and DCF fluorescence intensity per mg protein was calculated.

C2-ceramide treatment

Cell permeable ceramide (C2-ceramide) was purchase from Sigma (St Louis, MO). C2-ceramide was solved in DMSO (20mM) as stock solution. C2-ceramide was added to cell culture media to the final concentration of 10 μ M. The control groups were treated with the same volume of DMSO.

Ceramide measurement

After treatment, HaCaT cells were trypsinized from plates and the cell numbers were counted by Coulter Z1 particle counter (Beckman Coulter, Miami, FL). Cells were collected by

centrifuging at 1000 x g for 5 minutes. The supernant was discarded and the pellets were frozen at -80°C . Cellular ceramide concentrations were determined by thin layer chromatography (TLC) method [26]. The samples were lyophilized over night. Lipids were extracted using chloroform and methanol, 2:1, 1:1 and 1:2 successively for 1 hour each at room temperature. Combined extracts from each sample were dried under nitrogen. Samples were stored at -20° until analysis.

For each ceramide band, peak areas and the standard curve were used to calculate μg ceramide per sample. The results were expressed as micrograms ceramide per million cells.

Statistical analysis

Single factor ANOVA, followed by Student's *t*-test was used to compare statistical differences between means. Statistical significance was set at the $p \leq 0.05$ level.

Results

UVA irradiation caused accumulation of extracellular H_2O_2 in HaCaT cells

We found that both mRNA and protein level of AP-2 α increased after 5 J per cm^2 UVA irradiation (see Supplemental data). The change in steady state AP-2 α mRNA levels was similar to a previous study in which the mRNA level also showed a biphasic pattern [27]. We also found that UVA irradiation caused accumulation of extracellular H_2O_2 in the medium. This increase could be inhibited by direct addition of native catalase protein into the medium (Figure 1 A), whereas of boiled catalase did not inhibit the signal, indicating that the pHPA was specific for hydrogen peroxide as opposed to other organic peroxides (data not shown).

H_2O_2 directly induced the expression of AP-2 α in HaCaT cells

Incubation HaCaT cells with 200 μM H_2O_2 for 30 min caused a significant increase in the levels of both AP-2 α mRNA and protein (Figure 1 B and C).

Forced expression of GPx-1 abolished intracellular peroxide induced by UVA irradiation

Adenoviral infection of HaCaT cells with Ad-GPx-1 increased GPx-1 protein and activity. The background level of GPx-1 expression in HaCaT cells is relatively low. In vector control cells, GPx-1 protein was undetectable (Figure 2 A, lane 1). At 100 MOI infection, Ad-GPx-1 significantly increased the GPx-1 protein level (Figure 2 A, lane 2), as well as the activity (Figure 2 B). In addition, neither the protein level nor the activity of the adenovirally expressed GPx-1 were affected by UVA irradiation in these experiments. Ad-GPx-1 (100 MOI) increased the GPx-1 activity three-fold as determined by the GPx assay performed as described in Materials and Methods. The intracellular H_2O_2 concentrations were measured with DCFH-DA fluorescent probe and normalized to the protein concentration of cell lysate. The arbitrary fluorescence units per milligram protein were then normalized to the control groups. We found UVA irradiation not only increased extracellular H_2O_2 (Figure 1 A), but also increased the intracellular H_2O_2 about 75% more than the untreated control group (Figure 2 C). Moreover, over expression of GPx-1 inhibited the accumulation of H_2O_2 in the UVA irradiated group (Figure 2 C), as well as in the untreated group.

Overexpression of GPx-1 decreased UVA induced expression of AP-2 α

HaCaT cells were infected with 100 MOI Ad-GPx or Ad-BglII and exposed to 5 J/ cm^2 UVA, and nuclear proteins were extracted after 4 hours and 18 hours. AP-2 α protein levels as determined by western blotting increased at both time points in the control group, while there was no significant increase in GPx-1 group (Figure 3 A). Furthermore, in the control groups, AP-2 responsive CAT reporter activity increased about 2-3 fold 18 hours after UVA irradiation

compared to non-irradiated cells. In the GPx-1 over expressing group, although the basal level on AP-2 activity was slightly increased compared to the vector control groups, the UVA induced up-regulation was attenuated (Figure 3 B).

C2-ceramide induced AP-2 expression in HaCaT cells

Since ceramide was found be the key second messenger in the UVA induced AP-2 pathway [27], we wanted to determine whether HaCaT cells treated with cell permeable ceramide could also induce AP-2 transcription. HaCaT cells were treated with 10 μ M ceramide or DMSO (control). Total RNA and nuclear proteins were harvested 8 hours later. Quantitative RT-PCR (Figure 4 A) and western blot (Figure 4 B) showed that C2-ceramide significantly induced AP-2 expression.

GPx-1 abolished the increase of ceramide caused by H₂O₂ and UVA

HaCaT cells were infected with 100 MOI Ad-Bgl II or Ad-GPx-1 for 24 hours. Two groups of cells were then treated with 200 μ M H₂O₂ or 5 J per cm² UVA while the control groups remained untreated. We collected cells 4 hours after treatment to measure ceramide concentration. We found that both H₂O₂ and UVA radiation could induce ceramide (Figure 4 C) and overexpression of GPx-1 not only inhibited the ceramide generation in control groups, but also in H₂O₂ and UVA groups (Figure 4 C).

DISCUSSION

In our current study, we found that low dose UVA irradiation (5 J per cm²) induced AP-2 expression in immortalized human keratinocyte HaCaT cells. The biphasic induction pattern of both mRNA and protein was very similar to that previously observed in long-term cultured, normal human keratinocytes [12,28]. The UVA induced expression of AP-2 was likely to be at the level of transcription, since an AP-2 promoter fragment was readily transactivated in UVA exposed HaCaT cells in a promoter-reporter assay (supplemental data).

The biological effects of UVA are often ascribed primarily to singlet oxygen, however we found that hydrogen peroxide (H₂O₂) accumulation was also induced by UVA irradiation. H₂O₂ is not only involved in oxidant damage to cellular components, but also plays a central role in the signal transduction including oxidation of thiol residues on peroxiredoxins and protein tyrosine phosphatases. We found that UVA irradiation induced the generation of H₂O₂, and that H₂O₂ was capable of inducing the expression of AP-2 when applied directly to cells. Thus, not only UVA, but also H₂O₂ directly led to the increased expression of AP-2 in human keratinocytes.

In human and other mammalian cells, H₂O₂ is eliminated by catalase, glutathione peroxidases (GPx) and peroxiredoxins. H₂O₂ moves across plasma and peroxisomal membranes reversibly following first-order kinetics [29], and catalase is localized in peroxisomes. GPx-1, also called cytoplasmic GPx, is one of four isoforms of GPx. Although its main location was suggested to be in the cytoplasm, it has also been found in the nucleus [30]. This localization characteristic may make GPx-1 a better candidate than catalase for modifying cytoplasmic signal transduction pathway.

A change in cellular ceramide concentration is a downstream event after oxidative stress including UVA irradiation and H₂O₂ exposure. Elevated ceramide concentration is a characteristic feature of epidermal differentiation. The level of ceramide in keratinocytes has been shown to respond to UVA irradiation through a sequential triggering of enzymatic and non-enzymatic pathways [27]. It also has been shown that hydrogen peroxide-mediated oxidative stress modulates ceramide levels in lung epithelium [31]. H₂O₂ directly stimulated

sphingomyelin hydrolysis to ceramide through a neutral Mg^{2+} -dependent sphingomyelinase (nSMase) [31]. In this study, we showed that GPx-1 not only abolished the induction of AP-2 by UVA irradiation, but also inhibited the UVA induced production of ceramide, which may be the mechanism that GPx-1 inhibited AP-2 induction.

Ceramide has been shown to play a causal role in differentiation and apoptosis [31], and also has the ability to stimulate cell growth through ceramide-activated protein kinase (CAPK) and MAP kinase [32]. Further studies will be required to investigate the role of AP-2 upon UVA induced ceramide accumulation and its role on the biology of keratinocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

UVA irradiation of HaCaT cells caused hydrogen peroxide accumulation in the extracellular media. **A.** HaCaT cells were irradiated with UVA (5 J per cm²) and the media was changed to the pHPA working buffer for H₂O₂ measurement as described in Materials and Methods. To determine that the signal was due specifically to H₂O₂ and not other peroxides, 5000 U of catalase protein was added to the buffer. The fluorescent signals were normalized to the cell numbers for each group. * p<0.01 versus no UVA and catalase groups (n ≥ 3). **B.** AP-2α steady state mRNA levels were induced by H₂O₂. Total RNA was extracted and quantitative RT-PCR was performed to measure the relative level of AP-2α (normalized to β-actin as described). * p<0.05, † p<0.01 vs control group (n ≥ 3). **C.** AP-2α protein levels were also induced by H₂O₂. Nuclear proteins were extracted and subjected to western blot. GAPDH was used as a control for loading and transfer.

Figure 2.

Adenoviral GPx-1 induced overexpression of GPx-1 in HaCaT cells and abolished intracellular H_2O_2 . HaCaT cells were infected with either Ad-Bgl II or Ad-GPx-1. Cells were supplemented with 30 nM sodium selenite in the culture media. Total cellular proteins were harvested 24 hours later. For the UVA irradiated group, the cells received 5 J per cm^2 UVA radiation and were incubated for 4 hours before harvest. Ad-GPx-1 increased GPx-1 protein (**A**) and activity levels (**B**) in HaCaT cells. * $p < 0.05$ vs vector control ($n \geq 3$). **C.** GPx-1 abolished intracellular H_2O_2 . HaCaT cells were infected with either 100 MOI Ad-Bgl II or Ad-GPx-1. Twenty four hours later, UVA irradiation at a dose of 5 J per cm^2 was applied to the UVA groups. DCFH-DA was added to each plate as described in Materials and Methods for measuring peroxides. For the untreated control group, ‡ $p < 0.01$ vs vector only control; for the UVA irradiated group, * $p < 0.01$ vs untreated control; † $p < 0.001$ vs UVA irradiated vector control ($n \geq 3$).

Figure 3.

GPx-1 prevented UVA induced AP-2α expression. **A.** HaCaT cells were infected with 100 MOI of either Ad-Bgl II or Ad-GPx-1 for 24 hours. Each group received 5 J per cm² UVA irradiation. Nuclear proteins were harvested at 4 and 18 hours after UVA irradiation and applied to western blot assay. GAPDH was used as the control for loading and transfer. **B.** Five μg of an AP-2 responsive reporter construct (12 x AP-2 CAT) was co-transfected with 5 μg β-gal plasmid into each group of HaCaT cells in 60 mm plates. HaCaT cells were then infected with 100 MOI either Ad-Bgl II or Ad-GPx-1 for 24 hours. Each group received 5 J per cm² UVA irradiation. Total RNA was extracted 18 hours later and the ratios of CAT to β-gal mRNA levels in each group were measured with real-time RT-PCR. * p < 0.05 vs UVA- group (n ≥ 3).

Figure 4.

H₂O₂ and UVA irradiation induced ceramide accumulation in HaCaT cells that was abolished by GPx-1. HaCaT cells were treated with 10 μ M c2-ceramide or same volume of DMSO as a control. Total RNA and nuclear proteins were harvested 4 hours later. **A.** C2-ceramide increased AP-2 α steady state mRNA levels. AP-2 mRNA levels were measured with quantitative RT-PCR as described. Results are expressed as the ratio of AP-2 α to 18s and normalized to the DMSO control group. * $p < 0.05$, ($n \geq 6$). **B.** C2-ceramide induced AP-2 protein level. Nuclear protein was subjected to western blot and β -actin was used as the control for loading and transfer. **C.** GPx-1 inhibited UVA and H₂O₂ induced ceramide. HaCaT cells were treated with either 200 μ M H₂O₂ or 5 J per cm² UVA irradiation. Four hours later cells were counted and the ceramide concentrations were determined as described in Materials and Methods. The results were expressed as micrograms ceramide per million cells. * $p < 0.05$ vs GPx-1 groups; † $p < 0.05$ vs untreated control group ($n \geq 3$).