Gene Therapy (1999) 6, 1427–1437

http://www.stockton-press.co.uk/gt

Cellular redox state alters recombinant adeno-associated virus transduction through tyrosine phosphatase pathways

S Sanlioglu and JF Engelhardt
Department of Anatomy and Cell Biology and Department of Internal Medicine at the University of Iowa College of Medicine, Iowa City, Iowa, USA

Several types of environmental damage including UV, hydroxyurea and ionizing irradiation have been shown to augment rAAV transduction. Current hypotheses suggest that these environmental stimuli lead to the enhanced production and/or activation of cellular factors important in the conversion of single-stranded DNA genomes to expressible forms. However, the mechanisms of action are currently unknown. We hypothesized that reactive oxygen intermediates (ROI) may play a common role in the augmentation of rAAV transduction by these environmental stimuli. Our results demonstrate that treatment with hydrogen peroxide can give equivalent or greater levels of augmentation in rAAV transduction as that seen by hydroxyurea or UV irradiation. For all environmental stimuli, pretreatment with the hydroxyl radical ($H0^{\cdot}$) scavenger, N-acetyl-L-cysteine (NAC), completely blocked augmentation of rAAV transduction. Furthermore, using electron spin resonance spectroscopy (ESR), we demonstrated that both UV and H_2O_2 treatment of cell lines lead to the induction of $H0^{\cdot}$ radicals. Our results demonstrating that NaOV inhibits the augmentation of rAAV transduction following UV and H_2O_2 treatment, implicate $H0^{\cdot}$ radicals as modulators of tyrosine phosphatase pathways involved in rAAV transduction. Alterations in the cellular redox state and subsequent activation of tyrosine phosphatase pathways appear to alter the phosphorylation status of the previously identified single-stranded sequence binding protein (ssDBP), with reduced phosphorylation correlating with an enhancement in rAAV transduction. In summary, we conclude that the cellular redox state may play an important role in regulating rAAV transduction.

Keywords: *adeno-associated virus; reactive oxygen intermediates; tyrosine phosphatase; rAAV transduction*

Introduction

Recombinant AAV (rAAV) has demonstrated considerable promise as a gene therapy vector for certain organ systems such as muscle, brain and eye.[1–5] However, for reasons which are only partially understood, rAAV transduction is relatively inefficient in other cell types, including polarized airway epithelia and non-proliferating fibroblasts. Several hypotheses for tissue variability in rAAV transduction have been proposed, including the abundance of surface AAV receptor(s) and intracellular proteins (ie, D-sequence binding protein) important in the conversion of single-stranded AAV genomes to duplex expressible forms.[6,7] Several types of environmental damage, including UV, hydroxyurea (HU) and ionizing irradiation, have been shown to augment rAAV transduction in cell types exhibiting inefficient transduction. Current hypotheses suggest that these environmental stimuli lead to an enhanced production and/or activation of cellular factors important in the conversion of single-stranded DNA genomes to expressible forms.[8] Although the mechanisms involved are currently unknown, it has been hypothesized that DNA damage is a common feature of these environmental stresses.[9,10]

Cellular damage produced by HU, UV and ionizing irradiation involves the production of reactive oxygen intermediates (ROI).[11,12] In the case of HU, ROIs have been suggested to be intermediates in facilitating DNA damage, while UV and ionizing irradiation are generally thought to directly damage DNA without a requirement for ROIs. ROIs mainly consist of oxygen-centered molecules such as hydroxyl radicals ($H0^{\cdot}$), superoxide anions ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$).[10] In living cells, the initial products of reactive oxygen are generally thought to be H_2O_2 or superoxide anion.[13] Since superoxide anion is later converted into H_2O_2 by superoxide dismutase, H_2O_2 is regarded as a key substance in ROI-mediated cell injury.[14] Although H_2O_2 by itself is only slightly toxic, its byproduct hydroxyl radical ($H0^{\cdot}$) can oxidize DNA, proteins and membrane lipids, which lead to cellular injury.

In the current study, we hypothesized that ROIs may play a common role in the augmentation of rAAV transduction by these environmental stimuli. The activation of rAAV transduction, as detected with a EGFP reporter gene, was evaluated in Hela cells following treatment with UV irradiation, HU and H_2O_2. The results demonstrate that all of these conditions, including H_2O_2 treatment, significantly enhanced rAAV transduction. To

Correspondence: JF Engelhardt, Department of Anatomy and Cell Biology, University of Iowa College of Medicine, 51 Newton Road, Room 1–111 BSB, Iowa City, Iowa 52242, USA
Received 5 November 1998; accepted 12 April 1999

begin to investigate potential common mechanisms involved in redox modulation of rAAV transduction, we evaluated a potential signal transduction pathway which might alter the expression of cellular genes important in the conversion of rAAV genomes to expressible forms. Inhibition studies indicated that tyrosine phosphatase pathways might be an important aspect of ROI-mediated rAAV transduction. One such common pathway induced by ROIs is NF-κB.[12,15] NF-κB, well known as an immediate–early cellular response gene to environmental stress, regulates the expression of several cellular genes important in regulating the cell cycle, apoptosis and DNA repair (ie *p53* and *ref-1*). Interestingly, activation of T cell receptors has been shown to activate NF-κB through tyrosine kinase pathways, and NF-κB has been suggested to be an essential part of HIV LTR-mediated cellular transduction. Both UV irradiation and H_2O_2 treatment have also been reported to induce phosphorylation of EGF receptors through tyrosine kinase pathways.[16,17] Because of these observed links between tyrosine kinases, NF-κB activation, and the intracellular redox state, we hypothesized that activation of NF-κB mediated by both UV irradiation and H_2O_2 might be necessary for the enhanced cellular rAAV transduction. In order to understand the possible role of NF-κB in UV and H_2O_2 augmentation of rAAV transduction, a dominant mutant form of IkBα was used to inhibit NF-κB activation following UV and H_2O_2 treatment. Furthermore, examination of the phosphorylation status of ssD-BP by mobility shift assays indicated that cellular redox state alters the tyrosine phosphorylation status of ssD-BP favoring dephosphorylation. Results from these experiments demonstrate that the cellular redox state plays a vital role in the augmentation of rAAV transduction. These pathways appear to be mediated through NF-κB-independent tyrosine phosphatase pathways.

Results

Recombinant AAV transduction is enhanced by reactive oxygen intermediates and blocked by redox scavengers

Since several environmental stimuli known to augment rAAV transduction also enhance cellular levels of ROI, we tested whether H_2O_2 treatment could directly augment rAAV transduction using a EGFP reporter vector (AV.GFP3ori) in Hela cells. For comparison, Hela cells were also treated with HU (50 mm) or UV irradiated (25 J/m^2) immediately before rAAV infection. Following these treatments, Hela cells were infected with AV.GFP3ori virus at a multiplicity of infection (MOI) equal to 15 or 25 DNA particles per cell. The percentage of cells expressing EGFP was determined by FACS analysis 24 h following infection. As shown in Figure 1a and b, UV irradiation and HU treatment augmented AAV transduction by 10-fold at both MOI values tested. Studies evaluating levels of transduction at 48 and 72 h after infection were also performed and gave similar results (data not shown). These results confirm findings in previous reports.[18] Interestingly, 1 mm H_2O_2 also enhanced rAAV transduction 10-fold at MOI values of 25 particles per cell, but 18.2-fold at MOI values of 15 particles per cell. These data demonstrate that recombinant AAV transduction can be augmented with H_2O_2, and the level of augmentation is comparable with or better than that seen with UV and HU treatment.

To evaluate whether ROIs might be a common mediator responsible for augmentation of rAAV transduction by HU and UV treatment, we evaluated the ability of N-acetyl-L-cysteine (NAC), a known redox scavenger of $H0^{\cdot}$ radicals and H_2O_2, to attenuate UV and HU augmentation of rAAV transduction.[19] Results from these studies demonstrate that 50 mm NAC completely blocks the augmentation of rAAV transduction following H_2O_2, UV and HU treatment (Figure 1c, d and e). Inhibition was maximal at 50 mm NAC, but a dose-response of inhibition in rAAV transduction was seen at intermediate concentrations. These findings suggest that the common redox scavenger, NAC, can significantly inhibit augmentation of rAAV transduction induced by a number of environmental stimuli known to alter the redox state of cells.

UV irradiation and H_2O_2 treatment both produce hydroxyl radicals in cells

In order to determine if UV irradiation and H_2O_2 treatment produced the same type of ROIs in cells, electron spin resonance spectroscopy was performed (Figure 2). Reactive oxygen species were captured using DMPO (5, 5-dimetyl-1-pyrroline N-oxide) as explained in Materials and methods. Because of the nature of the assay, we need to UV irradiate cells in solution, and determine production of ROIs in minutes after UV irradiation. Therefore, higher doses of UV were needed in our assays (200 J/m^2 and 1000 J/m^2) to visualize radical formation. As seen in Figure 2, both UV irradiation and H_2O_2 treatment produced $OH^{\cdot}$ radicals (DMPO/OH adduct peaks are marked by asterisks in Figure 2). In contrast, control DMPO-treated Hela cells in the absence of UV or H_2O_2 treatment did not yield such radicals. This observation supports the notion that these agents produce similar types of ROIs which may be responsible for augmentation of rAAV transduction.

Augmentation of rAAV transduction by reactive oxygen intermediates is independent of transcriptional induction of the rAAV transgene cassette

In order to rule out the possibility that augmentation in rAAV transduction was due to increased transcriptional activity of the transgene reporter, Hela cells were treated with genotoxic agents as explained in Material and methods followed by transient transfection with a rAAV circular intermediate plasmid, p81, which contains the entire proviral genome.[20] As seen in Figure 3a, genotoxic agents did not increase the number of EGFP-positive cells compared with p81 alone. Rather, there was an apparent reduction in the number of EGFP-positive cells. This reduction in EGFP expression correlated with a reduction in mRNA on Northern blots (bottom panel in Figure 3a). In order to rule out nonspecific effects of genotoxic agents on DNA uptake/transfection efficiency, the order of treatment and transfection was reversed. In these experiments, cells were first transfected and then treated with genotoxic agents 24 h after the transfection. This maneuver would ensure that DNA transfection efficiencies were nearly equivalent for all condition before treatment with genotoxic agents. As seen in Figure 3b, the percentage of cells expressing EGFP remained unchanged at 24–48 h after treatment with genotoxic agents (Figure 3b, upper panel). Northern blotting using poly A m-RNA isolated from these cells also indicated

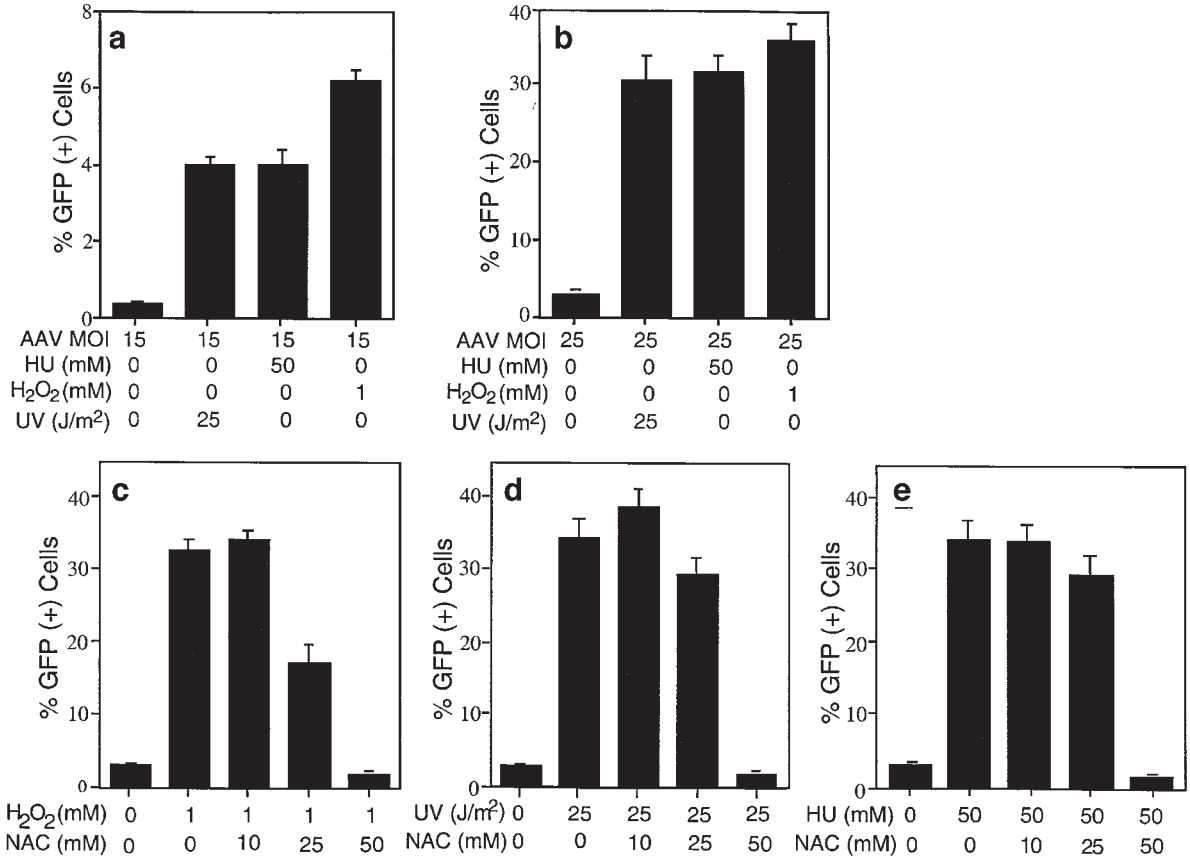

Figure 1 *Reactive oxygen intermediates augment rAAV transduction. Hela cells were treated with the indicated doses of HU, H_2O_2 or UV before infection with AV.GFP3ori virus at MOI values of 15 particles per cell (a) or 25 particles per cell (b). The percentage of cells expressing the GFP transgene was determined 24 h after infection by FACS analysis. Data represent the mean (+ s.e.m.) of six independent infections for each experimental data point. Hela cells were treated with increasing concentrations of NAC for 1 h before experimental treatment with H_2O_2, (c), UV (d) or HU (e), as described in Materials and methods. Following treatment with H_2O_2, UV or HU, cells were infected with AV.GFP3ori virus (MOI = 25 particles per cell). The percentage of cells expressing EGFP was determined at 24 h after infection by FACS analysis. Data represent the mean (± s.e.m.) of four independent infections for each experimental data point.*

that there was no increase in the amount of EGFP mRNA when cells were treated with these genotoxic agents (Figure 3b, bottom panel). Therefore, we have concluded that the augmentation in rAAV transduction is not due to transcription enhancement of the rAAV construct.

Redox modulated tyrosine phosphatase pathways are involved in rAAV transduction

Since both UV and H_2O_2 have been suggested to activate EGF receptors through tyrosine kinase pathways, we tested whether these pathways might also be involved in enhancing rAAV transduction. Hela cells were treated with a tyrosine phosphatase inhibitor (NaOV) at increasing doses before treatment with H_2O_2 and UV (Figure 4). Interestingly, NaOV treatment significantly reduced the augmentation in rAAV transduction induced by UV (Figure 4a) and H_2O_2 (Figure 4b) in a dose-dependent fashion. Therefore, signaling pathways that involve protein tyrosine phosphorylation are likely important mediators of rAAV transduction by these environmental stimuli. Given the commonality of redox modulation by UV, HU and H_2O_2, we sought to identify potential signal transduction cascade pathways that might be responsible for augmentation of rAAV transduction by these environmental stimuli. One such pathway, NF-κB, is well known to be modulated by the cellular redox state and specifically by H_2O_2 and UV irradiation.[15] Moreover, the redox scavenger NAC has been shown to block ROI-mediated NF-κB activation.[21–23] Interestingly, our results demonstrate that rAAV transduction mediated by H_2O_2, UV and HU can be inhibited by the same antioxidant NAC. Because tyrosine kinase pathways are linked to activation of NF-κB, and alterations in tyrosine kinase phosphorylation by NaOV also reduced rAAV transduction, we decided to investigate the NF-κB signaling pathway for potential involvement in ROI-mediated rAAV transduction.

Reactive oxygen intermediates and UV irradiation activate NF-κB pathways

To determine the time-course of NF-κB activation following H_2O_2 and UV irradiation in our model system, we performed electrophoretic mobility shift analysis (EMSA) using ^{32}P-labeled NF-κB oligos. TNFα (10 ng/ml), a potent NF-κB activator, was used as a positive control in this assay and demonstrated significant activation of NF-κB (Figure 5a). Hela cells were treated with either 1 mm H_2O_2 for 1 h or UV irradiation at 25 J/m^2, and nuclear extracts were prepared at different time-points following treatment. As seen in Figure 5a, NF-κB activation was

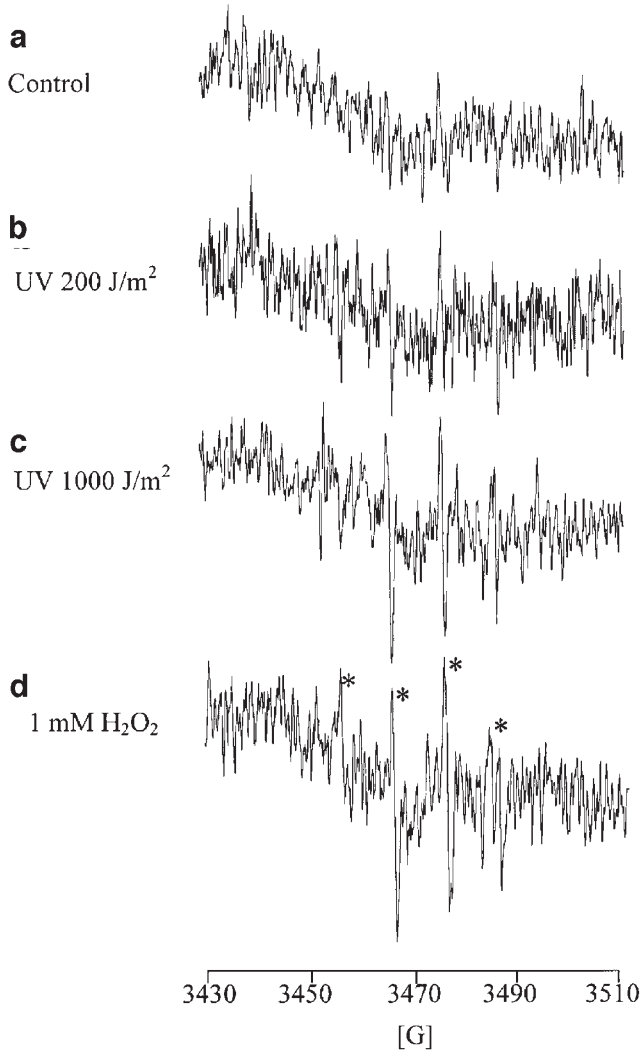

Figure 2 *ESR spectra of HO radical produced following UV irradiation and H_2O_2 treatment of Hela cells. Hela cells were trypsinized and resuspended at a density of 1×10^6 cells/ml in PBS and assayed in the presence of DMPO hydroxyl radical trapper under the indicated conditions as described in Materials and methods. DMPO was added immediately following UV irradiation and H_2O_2 treatment just before ESR measurements. Asterisks mark DMPO/OH adduct as indicated by four symmetrical peaks. Spectra shown are representative of at least two independent experiments.*

maximal after 1 h treatment with H_2O_2 TNFα or UV irradiation. To confirm the specificity of nuclear NF-κB DNA binding, competitive mobility shift assays were performed using either wild-type or mutated NF-κB sequences (Figure 5b). Only cold wild-type NF-κB oligos competed for binding of NF-κB. These results confirm the identity of the protein bound to shifted bands as NF-κB.

UV modulation of rAAV transduction is not controlled by NF-κB

To test whether NF-κB activation following UV exposure was required for activation of cellular pathways responsible for the augmentation of rAAV transduction, we evaluated the effects of co-expressing the dominant inhibitor IkBM before UV exposure and rAAV infection. Recombinant adenovirus, Ad.IkBM, was used as the shuttle vector for expressing this inhibitor. In these studies, adenoviral constructs expressing the dominant negative mutant form of IkB (Ad.IkBM) or *LacZ* gene (Ad.CMVLacZ) were infected into Hela cells at different MOI values (10, 50, 250, 1000 particles per cell). The following day, Hela cells were UV irradiated and nuclear extracts were prepared 1 h after treatment (optimal timepoint for activation of NF-κB). EMSA was performed in order to determine whether the expression of a dominant mutant form of IkB could block UV-induced NF-κB activation. As shown in Figure 6a, Ad.IkBM infected Hela cells at MOI values of 10 and 50 failed to block NF-κB activation following UV exposure. However, NF-κB activation was blocked in Hela cells infected with 250 and 1000 particles per cell of Ad.IkBM. As a control for nonspecific effects of adenovirus on NF-κB activation, Hela cells were also infected with Ad.CMVLacZ at 1000 particles per cell before UV irradiation. In these experiments, NF-κB activation was identical to that seen in uninfected cells (Figure 6a). In summary, these results demonstrate that expression of IkBM blocks UV-induced NF-κB activation pathways and that recombinant adenovirus, at the doses used in these studies, does not nonspecifically interfere with activation of this pathway.

We next sought to evaluate whether down-regulation of NF-κB activation following UV irradiation would reduce the augmentation of rAAV transduction. Hela cells were infected with either Ad.IkBM or Ad.CMVLacZ at 250 and 1000 particles per cell. At 24 h after infection with recombinant adenovirus, cells were UV irradiated and infected with AV.GFP3ori (15 particles per cell). EGFP transgene expression from the rAAV construct was analyzed in cells 24 h after AAV infection by FACS analysis. As seen in Figure 6b, the typical 10-fold induction in AAV transduction was detected when cells were irradiated at 25 J/m^2 UV in the absence of adenovirus. In cells infected with adenoviral vector alone, a small but noticeable induction in EGFP transgene expression was evident, correlating with the dose of virus. These effects are similar to those previously reported as E4-ORF6 augmentation of rAAV transduction.[24,25] However, when cells were infected with the Ad.IkBM construct at MOIs of 250 and 1000 particles per cell followed by UV irradiation, rAAV transduction rose 47-fold and 83-fold, respectively, as compared with cells infected with Ad.IkBM alone. This represented a four- to eight-fold increase in induction over UV alone. The reason for this higher level of transduction in the presence of both adenovirus and UV likely represents the combined effects of both UV and E4-ORF6 on rAAV transduction. Most importantly, when cells were infected with Ad.CMVLacZ virus followed by UV exposure, no significant differences in the level of rAAV transduction was seen when compared with cells treated with Ad.IkBM and UV. In summary, neither cells expressing the Ad.IkBM nor the Ad.CMVLacZ displayed a reduction in UV augmented rAAV transduction. From these data we conclude that the NF-κB pathway is not necessary for UV augmentation effects on rAAV transduction.

H_2O_2 modulation of rAAV transduction is not controlled by NF-κB

Conclusively to rule out that redox activation of NF-κB was not involved in pathways responsible for augmentation of rAAV transduction, we also tested whether inhibition of H_2O_2-induced NF-κB by the dominant IkBM

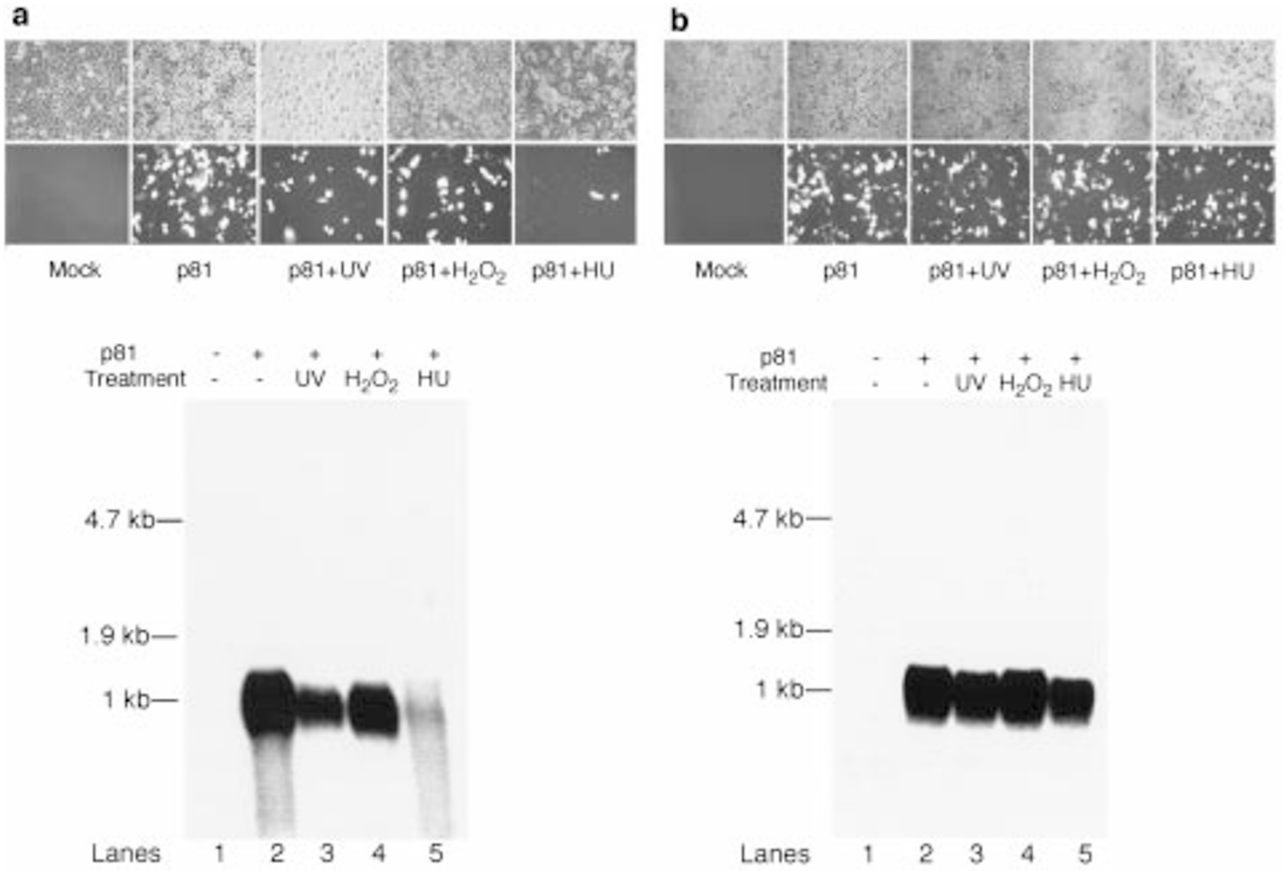

Figure 3 *Transcriptional effects of genotoxic agents on the rAAV transgene cassette. Hela cells were treated with genotoxic agents before (a) or following (b) liposome-mediated transfection of the rAAV proviral plasmid p81. Fluorescent and bright field photomicrographs taken at 24 h after transfection are given above each panel (representative of three to four experiments). Northern blot analysis of poly-A mRNA isolated at 24 h from these cells is given at the bottom of each panel. Lane 1, mock transfected cells; lane 2, untreated p81 transfected cells; lane 3, UV irradiated (25 J/m^2) p81 transfected cells; lane 4, H_2O_2-treated (1 mm) p81 transfected cells; lane 5, hydroxyurea-treated (50 mm) p81 transfected cells.*

would reduce H_2O_2-activated rAAV transgene expression. Since we observed optimal inhibition of UV-induced NF-κB activation at MOIs of 250 and 1000 particles per cell of Ad.IkBM, we have used only these two doses in this analysis. Nuclear extracts prepared from Hela cells infected with the Ad.IkBM exhibited complete inhibition of NF-κB activation pathway following H_2O_2 exposure (Figure 6c). In contrast, normal levels of NF-κB activation were seen in uninfected and cells infected with the control Ad.CMVLacZ construct (Figure 6c). These data confirm that H_2O_2-induced NF-κB DNA binding activity can be blocked by expression of the IkBM. We next evaluated the effect of IkBM expression on H_2O_2 augmentation of rAAV transgene expression. Studies were performed identically to those for UV irradiation (Figure 6b) with the exception that H_2O_2 exposure was substituted as the environmental stimuli. Findings evaluating H_2O_2 augmentation of rAAV EGFP expression were similar to those seen with UV (Figure 6d). No net difference in augmentation was seen in the presence of either Ad.IkBM or Ad.CMVLacZ. As in the UV experiments, these studies suggest that activation of NF-κB pathways are not necessary for augmentation in rAAV transduction seen following H_2O_2 exposure.

Cellular redox state alters the tyrosine phosphorylation status of ssD-BP

Previously, tyrosine dephosphorylation of a single-standed sequence binding protein (ssD-BP) has been correlated with the activation of rAAV transduction.[7,8] In order to test if cellular redox state alters the phosphorylation status of ssD-BP, a mobility shift assay using ^{32}P-labeled AAV D(–) sequence was performed. Interestingly, the ratio (r) of dephosphorylated (–P) to phosphorylated (+P) forms of ssD-BP was 2.6 in untreated Hela cells (Figure 7, lane 1). This number is significantly higher than that previously reported (r = 0.3) by Qing *et al*[8] for Hela cells. This may represent differences in assay conditions such as confluency or clonal variations in the Hela cell lines used. Our initial observation indicated only a small increase in the ratio of (–P)/(+P) forms of ssD-BP when cells were treated with 1 mm H_2O_2 or irradiated at 25 J/m^2 (from r = 2.6 to r = 3.2). However, increasing concentrations of H_2O_2 (50 mm) or UV (1000 J/m^2) resulted dephosphorylation of ssD-BP changing the ratio to r = 5.3 and r = 8, respectively (Figure 7). It is interesting to note that these are the same UV doses necessary to capture $H0^{\cdot}$ radicals induced by UV irradiation (Figure 2). NaOV treatment (tyrosine phos-

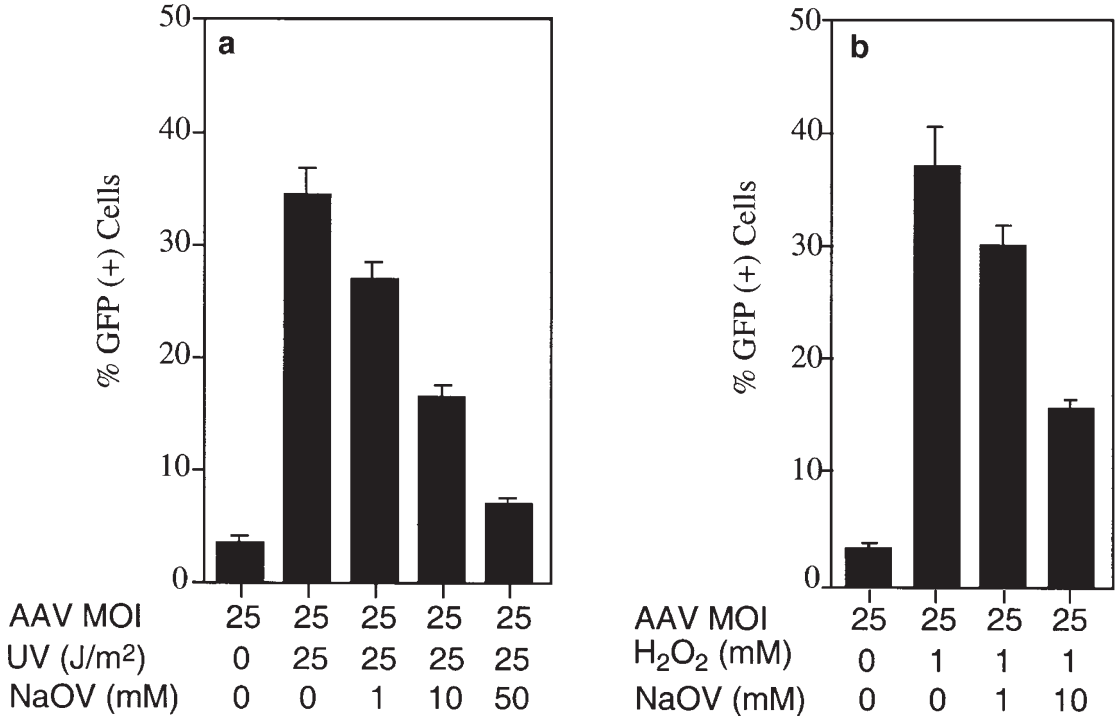

Figure 4 *Tyrosine phosphatase signaling pathways are in part responsible for ROI augmentation of rAAV transduction. The involvement of protein tyrosine phosphatase signaling pathways (PTP) in ROI-mediated rAAV transduction was evaluated in (a) and (b). Hela cells were treated with increasing doses of NaOV for 2 h before the treatment with H_2O_2 (b) or UV (a) as explained in Materials and methods. Results were analyzed 24 h following infection with rAAV at an MOI of 25 DNA particles per cell. Since 50 mm concentration of NaOV following treatment with 1 mm H_2O_2 but not UV was toxic for the cells, the corresponding values were omitted from (b). Data represent the mean (± s.e.m.) of four independent infections for each experimental data point.*

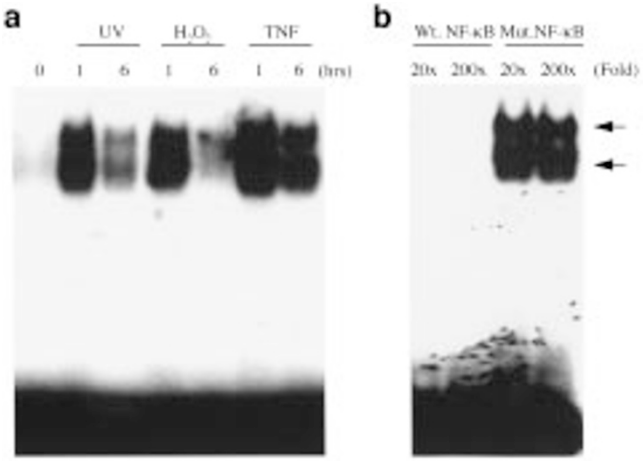

Figure 5 *Reactive oxygen intermediates activate NF-κB DNA binding. NF-κB DNA binding activity was evaluated in Hela cell nuclear extracts (15 μg) by electrophoretic mobility shift assays at 1 and 6 h following treatment with H_2O_2 (1 mm), UV (25 J/m²) or TNFα (10 ng/ml) (a). The specificity of NF-κB DNA binding was evaluated by competitive mobility shift assays (b) in the presence of cold wild-type (lanes 1 and 2) or mutant NF-κB (lanes 3 and 4) double-stranded DNA oligos. The probe used for binding in all lanes was ^{32}P-labeled wild-type NF-κB oligos. Cold competitors were used at 20-fold (20×) or 200-fold (200×) concentrations above the ^{32}P-labeled wild-type NF-κB oligos. Arrows indicate shifted NF-κB complexes and experiments are representative of three experiments.*

phatase inhibitor) significantly increased the level of (+P) form of ssD-BP (r = 0.7) as one might expect. While the induction of ROIs resulted in the dephosphorylation of ssD-BP leading to activation of rAAV transduction, NaOV treatment blocked this process leading to the reduction in rAAV transduction induced by UV and H_2O_2. Therefore, we concluded that cellular redox state decreases phosphorylation of ssD-BP and this effect can be blocked by the tyrosine phosphatase inhibitor NaOV. These data lend support to the importance of a redox-regulated tyrosine phosphatase pathway in rAAV transduction.

Discussion

ROIs are recognized as important modulators in a variety of signal transduction cascades which in turn alter gene expression patterns in response to environmental stress. UV irradiation, ionizing radiation and HU treatment have all been previously demonstrated to generate ROIs, cause DNA damage directly and modulate redox sensitive signal transduction pathways, such as p53, NF-κB, and JNK.[26–31] We hypothesized that ROIs may play a common role in the augmentation of rAAV transduction by these environmental stimuli. Findings that H_2O_2 treatment of Hela cells before rAAV infection gives rise to similar increases in transduction as seen with UV and HU treatment support this hypothesis. ESR studies demonstrating increased hydroxyl radical production in living cells following treatment with UV and H_2O_2 lend evidence for common ROI-mediated events. Furthermore,

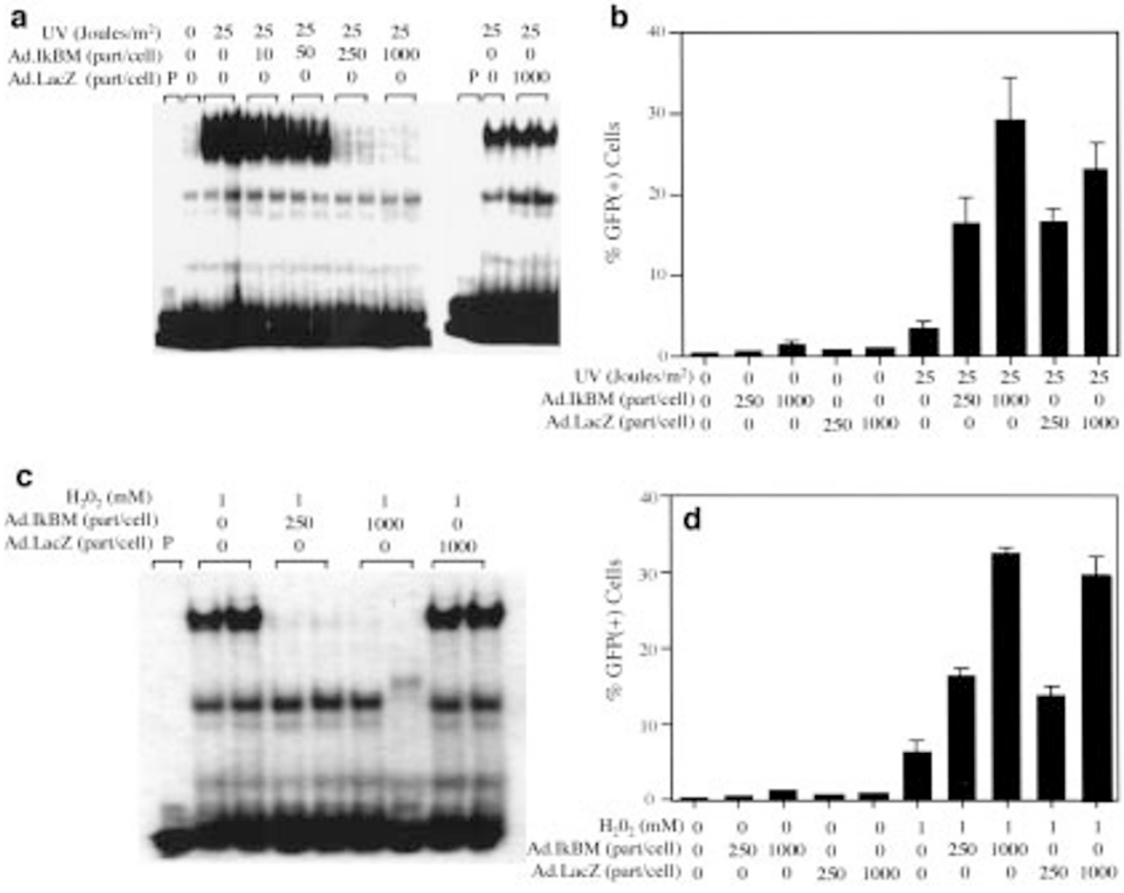

Figure 6 *UV-mediated NF-κB activation is blocked by Ad.IkBM. Hela cells were infected with recombinant adenovirus expressing the dominant mutant form of IkBα (Ad.IkBM) at MOI values of 10, 50, 250 and 1000 or the LacZ gene (Ad.CMVLacZ) at an MOI value of 1000. The following day, Hela cells were UV irradiated at 25 J/m². Nuclear extracts were prepared 1 h after UV irradiation and NF-κB DNA binding evaluated with ^{32}P-labeled wild-type NF-κB double-stranded oligos (a). The first lanes on both gels (marked P) represent free probe without added nuclear extract. Conditions for each experimental point including MOI of adenoviral infection (particles per cell) and UV dose (J/m²) are indicated above each lane. Nuclear extracts were prepared from two independent plates for each condition and mobility shift assays were performed twice to confirm the observations. The effect of IkBM expression on UV-induced rAAV transduction was evaluated in (b). Hela cells were infected with Ad.IkBM or Ad.CMVLacZ viruses 24 h before UV irradiation (25 J/m²) and infection with AV.GFP3ori (MOI = 15 particles per cell). The percentages of transgene expressing cells were evaluated at 24 h after infection by FACS analysis. Data represent the mean (± s.e.m.) of n = 6 independent points. H_2O_2-mediated NF-κB activation is blocked by Ad.IkBM (c). Hela cells were infected with Ad.IkBM or Ad.CMVLacZ for 24 h. The following day, Hela cells were treated with 1 mm H_2O_2 for 1 h. Nuclear extracts were prepared 1 h after H_2O_2 treatment and NF-κB DNA binding evaluated with ^{32}P-labeled wild-type NF-κB double-stranded oligos (c). The first lane (marked P) represents free probe without added nuclear extract. Conditions for each experimental point including MOI of adenoviral infection (particles per cell) and H_2O_2 dose (mm) are indicated above each lane. Nuclear extracts were prepared from two independent plates for each condition and mobility shift assays were performed twice to confirm the observations. Expression of IkBM does not alter H_2O_2-induced rAAV transduction (d). Hela cells were infected with Ad.IkBM or Ad.CMVLacZ viruses 24 h before treatment with H_2O_2 (1 mm for 1 h) and infection with AV.GFP3ori (MOI = 15 particles per cell). The percentages of transgene expressing cells were also evaluated at 24 h after AAV infection by FACS analysis. Data represent the mean (± s.e.m.) of n = 6 independent points.*

augmentation of rAAV transduction by H_2O_2, UV and HU, was blocked by the hydroxyl scavenger NAC. These results confirm the importance of cellular redox state in modulating rAAV transduction.

Two hypotheses could explain the mechanism of action by which ROIs augment rAAV transduction (Figure 8). First, ROIs could be direct effectors of DNA damage, which in turn activates pathways of DNA repair and increases the abundance and/or activity of enzymes responsible for converting single-stranded AAV genomes into duplex expressible forms. Alternatively, ROIs could directly influence signal transduction pathways responsible for altering the abundance or activity of factors necessary for AAV transduction. A common mechanism for redox modulation of signal transduction cascades is protein phosphorylation. Interestingly, H_2O_2 by itself and UV-induced ROIs have been shown to activate the epidermal growth factor receptor through a mechanism involving tyrosine phosphorylation.[16,17] With regards to AAV transduction, one obvious tyrosine phosphorylation-regulated pathway, which has been previously shown to influence AAV transduction, involves the D-sequence binding protein.[7,8] Previous studies have suggested that the level of phosphorylation of this ssD-BP correlates with second-strand DNA synthesis of rAAV. In the present study, we have demonstrated that cellular redox state modified by UV irradiation and H_2O_2 treatment modulate the phosphorylation status of ssD-BP in concordance with enhanced rAAV transduction. Inhibition studies using the tyrosine phosphatase inhibitors

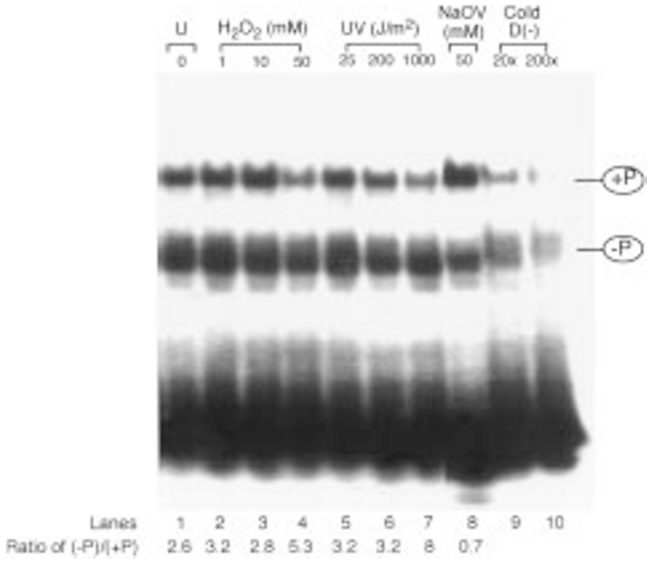

Figure 7 *Cellular redox state alters the phosphorylation status of ssD-BP. Hela cells were treated with genotoxic agents at the indicated doses above each lane as described in Materials and methods. Nuclear extracts were prepared from cells and electrophoretic mobility shifts assays performed with ^{32}P-labeled D(–) sequence DNA probes. Untreated (U) control nuclear extracts are shown in lane 1. Nuclear extracts from cells treated with increasing doses of H_2O_2 (lanes 2–4) and UV (lanes 5–7) are shown at 2 h after treatment. Cells were also treated with 50 mm NaOV for 2 h before preparation of nuclear extracts (lane 7). Competition experiments in lanes 9 and 10 represent untreated control nuclear extracts in the presence of 20-fold (20 ×) and 200-fold (200 ×) access of cold D(–) oligo. Phosphorylated (+P) and unphosphorylated (–P) bands are marked to the right of the gel as per previous identification.[7,8] The ratio of (–P)/(+P) was determined by densitometric analysis and is given below each lane.*

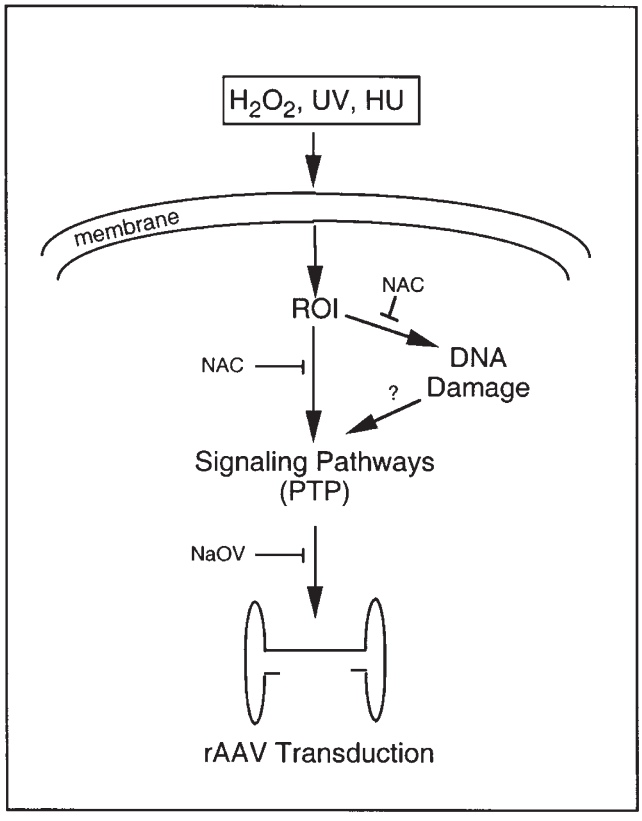

Figure 8 *A model for ROI-induced rAAV transduction. The proposed model attempts to explain several features of ROI-mediated rAAV transduction. For a detailed description of this model see the text. PTP, protein tyrosine phosphatase.*

NaOV suggest that UV and H_2O_2 redox modulation of ssD-BP phosphorylation likely occur at the level of tyrosine phosphatase pathways.

We next sought to evaluate potentially important signal transduction cascades which may lie proximal to activation of proteins directly associated with the genome conversion of rAAV. One such pathway known to be activated by both UV and H_2O_2 is NF-κB. Because of the importance of protein tyrosine kinase (PTK) pathways and ROIs in NF-κB activation, we tested whether NF-κB is important for rAAV transduction. Results demonstrated significant enhancement of NF-κB DNA binding following treatment by UV and H_2O_2. Furthermore, the antioxidant NAC has been demonstrated to block ROI-mediated NF-κB activation following ionizing radiation and H_2O_2 exposure.[21–23] Based on the findings that NAC also blocked ROI-mediated rAAV transduction in our assays and PTKs have been demonstrated to be involved in NF-κB activation, we investigated whether NF-κB activation could be directly responsible for redox augmentation of rAAV transduction. To this end, a dominant mutant form of IkB-α was expressed in Hela cells using recombinant adenovirus (Ad.IkBM). In this mutant, redox-mediated phosphorylation of IkB-α is blocked by mutations at key serine residues responsible for signaling ubiquitin degradation. Such mutants have been demonstrated to block NF-κB activation by TNF treatment. Although UV and H_2O_2 activation of NF-κB was successfully blocked by expression of Ad.IkBM, no effect was seen on the level of rAAV transduction as compared with Ad.CMVLacZ infected controls. These results suggest that H_2O_2 and UV-mediated enhanced AAV transduction is independent of NF-κB activation. However, because of the synergistic activation of rAAV transduction induced by ROIs and adenovirus, it is also possible that any subtle effect involving NF-κB and ROI-induced rAAV transduction might have been overwhelmed in this process.

Our data conclusively demonstrate that the cellular redox state significantly contributes to the enhancement of rAAV transduction resulting from a variety of environmental stresses. Protein phosphatase and / or kinase pathways independent of NF-κB activation appear to at least in part mediate these effects. A better understanding of mechanisms underlying redox augmentation of rAAV transduction may lead to more effective approaches for increasing the efficacy of gene transfer with this vector *in vivo*.

Materials and methods

Generation of recombinant AAV stocks

The cis-acting plasmid (pCisAV.GFP3ori) used for rAAV production was generated by subcloning the *Bsp*1201 / *Not*I fragment (743 bp) of the EGFP transgene from pEGFP-1 (Clontech, Palo Alto, CA, USA) between the CMV enhancer / promoter and SV40 polyA by blunt-end ligation. The CMV enhancer / promoter and SV40poly A sequences were derived from pcDNA3.1

(Invitrogen, Carlsbad, CA, USA). A 2.5 kb cassette containing β-lactamase and a bacterial replication origin from pUC 19 was blunt ligated down-stream of the GFP reporter cassette. The AAV ITR elements were derived from pSub201.[32] The entire plasmid contains a 4.7 kbp AAV component flanked by a 2 kb stuffer sequence derived from the luciferase gene of the pGL3-basic vector (Promega, Madison, WI, USA). Recombinant AAV stocks were generated by cotransfection of pCisAV.GFP3ori and pRep/Cap together with co-infection of recombinant Ad.CMVlacZ in 293 cells.[24] The rAV.GFP3ori virus was subsequently purified through three rounds of CsCl banding followed by heat inactivation of contaminating adenovirus at 60°C for 1 h, as previously described.[33] Typical yields from this viral preparation were 10^{12} DNA molecules per ml. DNA titers were determined by viral DNA slot blot hybridization against a EGFP P^{32}-labeled probe with copy number plasmid standards. The absence of helper adenovirus was confirmed by histochemical staining of rAAV infected 293 cells for β-galactosidase, and no recombinant adenovirus was found in 10^{10} particles of purified rAAV stocks. Additionally, rAAV viral stocks were essentially free from wt AAV as determined by immunocytochemical staining for Rep and Cap in an amplification assay as previously described.[33] The sensitivity of this assay was less then 1 wt AAV functional particle per 10^{10} recombinant AAV genomes.

Generation of Ad.LacZ and Ad.IkBM adenoviral stocks

Two recombinant adenoviral vectors expressing β-galactosidase (Ad.CMVLacZ) and the dominant mutant (S32A/S36A) form of IkBα (Ad.IkBM) were used for functional studies.[24,34] Recombinant adenoviral stocks were generated as previously described.[35] Viral stocks were stored in 10 mm Tris with 20% glycerol at –80°C. The particle titer of adenoviral stocks was determined by A_{260} readings and as typically 10^{13} DNA particles per ml. The functional titer of adenoviral stocks was determined by plaque titering on 293 cells. Typically, the particle per p.f.u ratios were equal to 25.

Assays for augmentation of rAAV transduction

Hela cells were plated at a density of 1×10^6 cells/35 mm plate. Several treatment groups were evaluated, including (1) control untreated, (2) HU treatment, (3) UV treatment, and (4) H_2O_2 treatment. In the case of HU treatment, 50 mm HU was supplemented to the media at 6 h after plating of cells and continued overnight for a total of 18 h before rAAV infection. Hela cells treated with H_2O_2 were incubated in 1 mm H_2O_2 for 1 h just before rAAV infection. Treatment with UV irradiation at 25 J/m^2 was also performed just before rAAV infection. Infection of cells with AV.GFP3ori virus (MOI = 15 and 25 DNA particles per cell) was performed at 24 h after plating (at confluency) for all conditions. Twenty-four hours after infection, FACS analysis was used to determine the percentage of cells expressing EGFP. Dead cells were stained by propidium iodide (PI) before FACS and were excluded from the data analysis. For NAC inhibition studies, Hela cells were plated at a density of 1×10^6 cells/35 mm plate and were treated with NAC (10 mm, 25 mm or 50 mm) for 1 h before treatment with HU (50 mm), UV (25 J/m^2) or H_2O_2 (1 mm). Cells were washed with media following NAC incubation, and the times of HU, UV and H_2O_2 treatment were identical to experiments performed in the absence of NAC treatment. Before infection with AV.GFP3ori virus (MOI = 25 particles per cell), cells were again washed with media to remove HU or H_2O_2. The percentage of cells expressing EGFP was determined 24 h after infection by FACS analysis. For studies that involved sodium orthovanadate (NaOV), Hela cells were treated with this inhibitors at indicated doses for 2 h and washed with PBS several times before treatment with genotoxic agents and infection with rAAV. All chemicals (NAC, HU, NaOV) used in these assays were purchased from Sigma, St Louis, MO, USA.

Electron spin resonance spectroscopy (ESR)

ESR was used to trap reactive oxygen species produced in cells following UV exposure or H_2O_2 treatment. ESR spectra were obtained at room temperature using a Bruker model EMX ESR spectrometer (Bruker, Karlsruhe, Germany) equipped with a TM_{110} cavity (University of Iowa, IA, USA). Instrument settings were as follows: receiver gain, 1×10^6; microwave power, 40 milliwatts; modulation frequency, 100 kHz; modulation amplitude, 1.0 G; sweep rate, 1.4 G/s. Briefly, cells were either UV irradiated at different doses (50–1000 J/m^2) or treated with H_2O_2 (1 mm and 10 mm). UV and H_2O_2 treatment were performed on trypsinized cells suspended in PBS at a density of 2×10^6 cells/ml. Immediately following treatment the hydroxyl radical trapper DMPO (5, 5-dimetyl-1-pyrroline N-oxide, oxygen centered radical trapper) was added to cells at a final concentration of 25 mm. Under conditions of H_2O_2 treatment, cells were washed with PBS before the addition of DMPO. Samples were immediately subjected to ESR spectroscopy following treatment with UV and H_2O_2.

Transfection and m-RNA isolation procedure

Confluent monolayers of Hela cells in 35 mm plates were treated with 50 mm hydroxyurea overnight, 1 mm H_2O_2 for 1 h, or UV irradiated at 25 J/m^2 before transfection with 3 μg of p81 rAAV circular intermediate. The p81 construct has been previously described and contains the full-length proviral genome.[20] All the transfections were performed using Lipofectamine reagent (Life Technologies, Gaithersburg, MD, USA) in triplicate. At 24 h after transfection, EGFP expression was examined under a fluorescent microscope before harvesting of cells for preparation of poly-A mRNA using a Oligotex Direct mRNA Mini Kit (Qiagen, Valencia, CA, USA). As an alternative approach, the order of treatment with genotoxic agents and transfection was reversed. In this case genotoxic agents were added at 24 h after transfection and poly-A mRNA purified 24 h after genotoxic treatment.

Northern blotting

Three micrograms of poly-A mRNA were resolved in a 1% agarose gel containing 6.3% formaldehyde and transferred to a Hybond-N nylon membrane (Amersham, Piscataway, NJ, USA). The filter was hybridized with a ^{32}P-labeled EGFP fragment isolated from *Eco*RI/*Not*I-restricted pEGFP-1 vector (Clontech, Palo Alto, CA, USA). Filters were washed in 0.2 × SSC at 65°C and exposed to X-ray overnight at –80°C.

Electrophoretic mobility shift assays (EMSA)

Nuclear extracts were prepared according to the procedure published by Andrews and Faller.[36] Briefly, a con-

fluent 100 mm plate of Hela cells was first washed with PBS and then scraped into a 1.5 ml centrifuge tube with 1 ml PBS. Cells were pelleted for 10 s and then resuspended in 400 μl of cold Hepes buffer (10 mm Hepes pH 7.9, 1.5 mm $MgCl_2$, 10 mm KCl, 0.5 mm DTT and 0.2 mm PMSF). Following incubation on ice for 10 min and vortexing for 10 s, cells were pelleted again. The supernatant was discarded and the nuclear pellet was resuspended in 100 μl of storage buffer (20 mm Hepes pH 7.9, 25% glycerol, 420 mm NaCl, 1.5 mm MgCl, 0.2 mm EDTA, 0.5 mm DTT and 0.2 mm PMSF). After a second incubation on ice for 20 min, the sample was centrifuged briefly and the supernatant transferred into a new microcentrifuge tube. Samples were stored at –150°C. The complementary oligo sequences used for double-stranded probes were as follows: NF-κB wild-type, EL-318: 5′-CCAGAGGGGAC TTTCCA-3′ and EL-319: 5′-ACGGCTGGAAAGTCCCC-3′; and NF-κB mutant, EL-328: 5′CCAGAGCTCAC TTTCAA-3′ and EL-329: 5′-ACGGCTTGAAAGTGAGC-3′ (Gibco-BRL, Rockville, MD, USA). Briefly, complementary, phosphorylated oligos were annealed, ends filled in the presence of α-^{32}P dCTP using Klenow, and the probe was purified from a polyacrylamide gel.

The procedure for measurement of single-stranded sequence binding protein activity (ssD-BP)

Measurements for ssD-BP activity were performed as previously described.[7,8] In brief, (^{32}P-labeled oligo representing the D(–) sequence of rAAV (AGGAACCCCTA GTGATGGAG) was used to measure the phosphorylation status of ssD-BP in Hela cells. For these experiments, Hela cells were treated with H_2O_2 (1 mm, 10 mm and 50 mm) for 1 h, UV (25, 200 and 1000 J/m^2) followed by a 1 h incubation, or NaOV (50 mm) for 2 h, before preparation of nuclear extracts. Nuclear isolation procedures and electrophoretic mobility shift assays were performed as described above. The ratio of dephosphorylated (–P) to phosphorylated (+P) forms of ssD-BP was determined by densitometric analysis using a QS30 program from PDI company (Huntington Station, NY, USA).

Inhibition of NF-κB activation using a recombinant adenovirus encoding the dominant mutant inhibitor IkBM

Hela cells were plated at a density of 3×10^5 cells/35 mm plate. On the following day, Hela cells were infected with increasing doses of either Ad.IkBM or Ad.CMVLacZ virus. Twenty-four hours after the infection, Hela cells were treated with UV (25 J/m^2) or H_2O_2 (1 mm) and nuclear extracts were prepared at 1 h after treatment (the optimal time-course for NF-κB induction). NF-κB binding activity was evaluated by mobility shift assays using wild-type NF-κB oligo probes. For studies evaluating rAAV infection, adenovirally infected Hela cells were treated with H_2O_2 or UV irradiated immediately before infection with AV.GFP3ori virus at an MOI of 15 DNA particles per cell. The percentage of cells expressing EGFP was determined by FACS analysis 24 h following rAAV infection.

Acknowledgements

The work described here was supported by NIH R01 DK/HL58340 (JFE) and the Gene Therapy Center supported by NIDDK (DK54759) and the Cystic Fibrosis Foundation. We also gratefully acknowledge Dr Larry Oberley for his helpful discussions on redox mechanisms and Dr Gary R Buettner, ESR facility at the University of Iowa.

References

1 Bennett J, Duan D, Engelhardt JF, Maguire AM. Real-time, noninvasive *in vivo* assessment of adeno-associated virus-mediated retinal transduction. *Invest Ophthalmol Vis Sci* 1997; **38**: 2857–2863.
2 Ali RR *et al.* Gene transfer into the mouse retina mediated by an adeno-associated viral vector. *Hum Mol Genet* 1996; **5**: 591–594.
3 Xiao X, Li J, Samulski RJ. Efficient long-term gene transfer into muscle tissue of immunocompetent mice by adeno-associated virus vector. *J Virol* 1996; **70**: 8098–8108.
4 Fisher KJ *et al.* Recombinant adeno-associated virus for muscle directed gene therapy. *Nature Med* 1997; **3**: 306–312.
5 Kaplitt MG *et al.* Long-term gene expression and phenotypic correction using adeno-associated virus vectors in the mammalian brain. *Nat Genet* 1994; **8**: 148–154.
6 Summerford C, Samulski RJ. Membrane-associated heparan sulfate proteoglycan is a receptor for adeno-associated virus type 2 virions. *J Virol* 1998; **72**: 1438–1445.
7 Qing K *et al.* Role of tyrosine phosphorylation of a cellular protein in adeno-associated virus 2-mediated transgene expression. *Proc Natl Acad Sci USA* 1997; **94**: 10879–10884.
8 Qing K *et al.* Adeno-associated virus type 2-mediated gene transfer: correlation of tyrosine phosphorylation of the cellular single-stranded D sequence-binding protein with transgene expression in human cells *in vitro* and murine tissues *in vivo*. *J Virol* 1998; **72**: 1593–1599.
9 Musonda CA, Chipman JK. Quercetin inhibits hydrogen peroxide (H2O2)-induced NF-kappa B DNA binding activity and DNA damage in HepG2 cells. *Carcinogenesis* 1998; **19**: 1583–1589.
10 Zhang X *et al.* Identification of possible reactive oxygen species involved in ultraviolet radiation-induced oxidative DNA damage. *Free Radic Biol Med* 1997; **23**: 980–985.
11 Devary Y, Rosette C, DiDonato JA, Karin M. NF-kappa B activation by ultraviolet light not dependent on a nuclear signal. *Science* 1993; **261**: 1442–1445.
12 Schreck R, Rieber P, Baeuerle PA. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J* 1991; **10**: 2247–2258.
13 Sasaki K, Bannai S, Makino N. Kinetics of hydrogen peroxide elimination by human umbilical vein endothelial cells in culture. *Biochim Biophys Acta* 1998; **1380**: 275–288.
14 Oredsson S, Plate G, Qvarfordt P. Experimental evaluation of oxygen free radical scavengers in the prevention of reperfusion injury in skeletal muscle. *Eur J Surg* 1994; **160**: 97–103.
15 Miller SC *et al.* Regulation of NF-kappa B and HIV-1 LTR activity in mouse L cells by ultraviolet radiation: LTR trans-activation in a nonirradiated genome in heterokaryons. *Exp Cell Res* 1997; **230**: 9–21.
16 Huang RP, Wu JX, Fan Y, Adamson ED. UV activates growth factor receptors via reactive oxygen intermediates. *J Cell Biol* 1996; **133**: 211–220.
17 Gamou S, Shimizu N. Hydrogen peroxide preferentially enhances the tyrosine phosphorylation of epidermal growth factor receptor. *FEBS Lett* 1995; **357**: 161–164.
18 Russell DW, Alexander IE, Miller AD. DNA synthesis and topoisomerase inhibitors increase transduction by adeno-associated virus vectors. *Proc Natl Acad Sci USA* 1995; **92**: 5719–5723.
19 Kawada N, Seki S, Inoue M, Kuroki T. Effect of antioxidants, resveratrol, quercetin, and N-acetylcysteine, on the functions of cultured rat hepatic stellate cells and Kupffer cells. *Hepatology* 1998; **27**: 1265–1274.
20 Duan D *et al.* Formation of adeno-associated virus circular genomes is differentially regulated by adenovirus E4-ORF6 and E2a gene expression. *J Virol* 1999; **73**: 161–169.

21 Blackwell TS *et al. In vivo* antioxidant treatment suppresses nuclear factor-kappa B activation and neutrophilic lung inflammation. *J Immunol* 1996; **157**: 1630–1637.
22 Kikumori T *et al.* Activation of transcriptionally active nuclear factor-kappa B by tumor necrosis factor-alpha and its inhibition by antioxidants in rat thyroid FRTL-5 cells. *Endocrinology* 1998; **139**: 1715–1722.
23 Sprong RC *et al.* Low-dose N-acetylcysteine protects rats against endotoxin-mediated oxidative stress, but high-dose increases mortality. *Am J Respir Crit Care Med* 1998; **157**: 1283–1293.
24 Fisher KJ *et al.* Transduction with recombinant adeno-associated virus for gene therapy is limited by leading-strand synthesis. *J Virol* 1996; **70**: 520–532.
25 Ferrari FK, Samulski T, Shenk T, Samulski RJ. Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J Virol* 1996; **70**: 3227–3234.
26 Flohe L *et al.* Redox regulation of NF-kappa B activation. *Free Radic Biol Med* 1997; **22**: 1115–1126.
27 Hirota K *et al.* AP-I transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc Natl Acad Sci USA* 1997; **94**: 3633–3638.
28 Lo YYC, Wong JMS, Cruz TF. Reactive oxygen species mediate cytokine activation of c-Jun NH2-terminal kinases. *J Biol Chem* 1996; **271**: 15703–15707.
29 Milne DM, Campbell LE, Campbell DG, Meek DW. p53 is phosphorylated *in vitro* and *in vivo* by an ultraviolet radiation-induced protein kinase characteristic of the c-Jun kinase, JNK1. *J Biol Chem* 1995; **270**: 5511–5518.
30 Irani K *et al.* Mitogenic signaling mediated by oxidants in Ras-transformed fibroblasts (see comments). *Science* 1997; **275**: 1649–1652.
31 Jayaraman L *et al.* Identification of redox/repair protein Ref-1 as a potent activator of p53. *Genes Dev* 1997; **11**: 558–570.
32 Samulski RJ, Chang LS, Shenk T. A recombinant plasmid from which an infectious adeno-associated virus genome can be excised *in vitro* and its use to study viral replication. *J Virol* 1987; **61**: 3096–3101.
33 Duan D, Fisher KJ, Burda JF, Engelhardt JF. Structural and functional heterogeneity of integrated recombinant AAV genomes. *Virus Res* 1997; **48**: 41–56.
34 Iimuro Y *et al.* NF-kappa B prevents apoptosis and liver dysfunction during liver regeneration. *J Clin Invest* 1998; **101**: 802–811.
35 Engelhardt JF *et al.* Direct gene transfer of human CFTR into human bronchial epithelia of xenografts with E1-deleted adenoviruses. *Nat Genet* 1993; **4**: 27–34.
36 Andrews NC, Faller DV. A rapid micropreparation technique for extraction of DNA-binding proteins from limiting numbers of mammalian cells. *Nucleic Acids Res* 1991; **19**: 2499.