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Oxidative Stress Increases A_1 Adenosine Receptor Expression by Activating Nuclear Factor κB

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ABSTRACT

The A_1 adenosine receptor (A_1AR) contributes to the cytoprotective action of adenosine under conditions known to generate reactive oxygen species (ROS). Pharmacological manipulation of A_1AR expression has been shown to modulate this cytoprotective role. In this study, we provide evidence that ROS generated could increase the expression of the A_1AR and thereby offset the detrimental effects of ROS. Incubation of DDT_1MF-2 smooth muscle cells with ROS-generating chemotherapeutic agents, such as cisplatin (2.5 μM) or H_2O_2 (10 μM), elicited an increase in A_1AR expression within 24 hr. The induction by H_2O_2 was reduced by the ROS scavenger catalase but not superoxide dismutase. Inhibition of nuclear factor κB (NFκB) by pyrrolidine dithiocarbamate (200 μM), dexamethasone (100 nM), or genistein (1 μM) abrogated the cisplatin-mediated increase in A_1AR. Cisplatin promoted rapid translocation of NFκB (but not AP-1) to the nucleus, as detected by electrophoretic mobility shift assays and by Western blotting. A putative NFκB sequence in the A_1AR promoter effectively competed with labeled κB probe for binding in nuclear preparations derived from DDT_1MF-2 cells. Transient transfection of DDT_1MF-2 cells with the A_1AR promoter coupled to firefly luciferase reporter gene led to cisplatin-inducible and pyrrolidine dithiocarbamate-sensitive luciferase activity, suggesting the presence of functional NFκB binding site(s) in the A_1AR promoter sequence. Treatment of cells with (*R*)-phenylisopropyladenosine (1 μM), an agonist of the A_1AR, reduced cisplatin-mediated lipid peroxidation, which was reversed after blockade of the A_1AR. These data suggest that ROS can increase the expression of the A_1AR by activating NFκB regulatory site(s) on this gene and thereby enhance the cytoprotective role of adenosine.

Adenosine, released under conditions of oxidative stress, confers cytoprotection in the cardiovascular and central nervous systems by activating cell surface adenosine receptors. This nucleoside provides rapid recovery of ventricular functioning induced after ischemia and reperfusion (Babbit *et al.*, 1989) and can effect a degree of protection similar to that observed after preconditioning (Liu *et al.*, 1991). In the central nervous system, adenosine and adenosine analogs protect against transient ischemia (Rudolphi *et al.*, 1992). Rats treated with caffeine to increase A_1AR expression in the brain were more resistant to ischemia, underscoring a cytoprotective role of this receptor subtype (Rudolphi *et al.*, 1989). In contrast, down-regulation of the A_1AR by prolonged agonist treatment exacerbated the damage created by a subsequent ischemic episode (Von Lubitz *et al.*, 1994). Several mechanisms contributing to the cytoprotective role of adenosine have been proposed. Adenosine hyperpolarizes neurons by activating K^+ conductance and inhibits Ca^{2+} influx into the nerve terminal (Rudolphi *et al.*, 1992). These two functions probably account for its ability to reduce the release of excitatory amino acids. Activation of A_1AR depresses neuronal firing rate and decreases neuronal metabolism, thereby promoting the preservation of ATP during ischemia (Rudolphi *et al.*, 1992).

Chemotherapeutic agents such as doxorubicin, daunomycin, mitoxantrone, bleomycin, and cisplatin promote oxidative stress by generating ROS (Powis, 1991). Anthracyclines, such as doxorubicin and daunorubicin, produce superoxide anions through redox cycling of these compounds in the mitochondria and hydroxyl radicals after reaction of superoxide anions with iron. The generation of ROS likely contributes to both the antitumor effects and side effects associated with these agents by potentiating DNA alkylation and strand breaks (Berlin and Haseltine, 1981). In fact, the generation of ROS may account for cisplatin-induced ototoxicity and neph-

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ABBREVIATIONS: A_1AR, A_1 adenosine receptor; APNEA, N^6-2-(4-amino-3-phenyl)ethyladenosine; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; DPCPX, 8-cyclopentyl-1,3-dipropylxanthine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MDA, malondialdehyde; NFκB, nuclear factor κB; PDTC, pyrrolidine dithiocarbamate; ROS, reactive oxygen species; (*R*)-PIA, (*R*)-phenylisopropyladenosine

rotoxicity and the cardiotoxicity produced by doxorubicin and daunorubicin (Myers *et al.*, 1977; Powis, 1991).

In the present study, we provide evidence that chemotherapeutic agents that generate ROS can directly influence A_1AR expression through activation of NFκB. Such a pathway provides an efficient mechanism by which the cell could counter the detrimental effects of ROS generated by chemotherapeutic agents or during ischemia.

Experimental Procedures

Cell cultures and radioligand binding. DDT_1MF-2 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 5% calf serum. Cells were maintained as a monolayer and detached in ice-cold phosphate-buffered saline containing 5 mM EDTA. The cells were then lysed in 10 mM Tris·HCl buffer, pH 7.4, containing 5 mM EDTA, 10 μg/ml soybean trypsin inhibitor, 10 μg/ml benzamidine, and 2 μg/ml pepstatin and homogenized briefly by Polytron homogenizer. Membranes were obtained by differential centrifugation, which involved centrifugation of the homogenates at 1,000 × *g* for 10 min followed by centrifugation of the supernatant at 40,000 × *g* for 15 min. The final pellet was resuspended in 50 mM Tris·HCl buffer, pH 7.4, 10 mM $MgCl_2$, and 1 mM EDTA, containing protease inhibitors (described above) and pretreated with adenosine deaminase (5 units/ml) to eliminate endogenous adenosine. Quantification of A_1AR was performed using the antagonist [^{3}H]DPCPX or the agonist radioligand ^{125}I-APNEA. ^{125}I-APNEA was diluted 10-fold with [I]APNEA to enable use of higher effective concentrations of the radioligand. Membrane preparations (approximately 80 μg of protein) were incubated with the radioligands in the absence or presence of 1 mM theophylline (to define nonspecific binding) for 1 hr at 37°. Samples were then filtered through polyethyleneimine-treated Whatman GF/B glass fiber filters, washed with 10 ml of ice-cold Tris buffer, and radioactivity determined by gamma or β counting. Saturation curves were analyzed by a computer-assisted curve-fitting program (Hancock *et al.*, 1979; DeLean *et al.*, 1982).

Western blotting. For quantifying the A_1AR, cell membranes were solubilized in 1% CHAPS buffer, desalted into 0.05% CHAPS, and 100–200 μg of protein samples were used for performing sodium dodecyl sulfate polyacrylamide gel electrophoresis. NFκB and c-Rel were quantified in nuclear extracts. Proteins were transferred to nitrocellulose membranes, blocked in Blotto buffer (130 mM NaCl, 2.7 mM KCl, 1.8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1% NaN_3, 0.1% Triton-X 100, and 5% low-fat skim milk) for 2 hr and then incubated with the primary antibody at 4° overnight. Polyclonal antibody for the A_1AR was obtained from Alpha Diagnostic International (San Antonio, TX), and polyclonal antibodies for NFκB and c-Rel were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). After five washes in blocking solution, blots were incubated with ^{125}I-labeled goat antirabbit IgG for 2 hr at room temperature, washed five times, and exposed to Kodak XAR film at −70° for about 48 hr. Quantification of the gels (^{125}I counts) was performed using a PhosphorImager (GS-250 Molecular Imager; BioRad, Hercules, CA) and by cutting and counting the labeled protein bands.

Electrophoretic mobility shift assay. Nuclear extracts were incubated with double stranded specific κB oligonucleotide (5′-ATGTGAGGGGACTTTCCCAGGC-3′) (Sen and Baltimore, 1986). Similar electrophoretic mobility shift assays were performed using a labeled oligonucleotide probe (5′-CGCTTGATGAGTCAGCCGGAA-3′) for the AP-1 transcription factor binding sequence. Incubations were performed at room temperature for 30 min in a total volume of 15 μl of buffer containing 12% glycerol, 12 mM HEPES-NaOH (pH 7.9), 60 mM KCl, 1 mM EDTA, and 1 mM dithiothreitol, 1.0 μg of poly(dI-dC), and 10,000 cpm of the labeled probe. The DNA-protein complexes were resolved on nondenaturing 5% polyacrylamide gels, performed with 0.5 × Tris/borate/EDTA buffer (4.5 mM Tris, 4.5 mM boric acid, 0.1 mM EDTA, pH 8.0). The fold increase in NFκB expression was determined with a PhosphorImager using background substract.

Luciferase assay. Cells were grown to a density of about 20–40% confluency and transfected with a mixture containing 100–250 ng of plasmid DNA, 500–650 ng of carrier DNA, 3 μl/g DNA of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulfate (lipofectin) in a volume of 50 μl Opti-MEM (GIBCO BRL, Gaithersburg, MD). The mixtures were allowed to stand for 45–60 min at room temperature before being added to the culture plate. After ~6 hr, regular media (Dulbecco's modified Eagle's medium, 10% fetal bovine serum) were added to the plate and it was returned to the incubator for 24 hr. For luciferase assays, cells were then lysed by using 50 μl of Reporter lysis buffer (Promega, Madison, WI) and centrifuged at 4° in a microcentrifuge at 12,000 × *g*. The extract was used immediately or stored at −70°. Twenty microliters of cell extract (supernatant) was mixed with 100 μl of Luciferase assay reagent (Promega) at room temperature and the chemiluminescent signal determined in a luminometer (1 min counts).

Protein determination and statistical analysis. The level of protein in samples was determined by the established method (Bradford, 1976), using bovine serum albumin to prepare standard curves. Statistical differences were determined by the analysis of variance, followed by *post hoc* analysis using the Tukey's test.

Results and Discussion

DDT_1MF-2 smooth muscle cells have been used previously as a model system for studying A_1AR (Ramkumar *et al.*, 1991). Treatment of DDT_1MF-2 cells with cisplatin (2.5 μM) produced an approximately 2-fold increase in A_1AR expression, determined by the antagonist radioligand [^{3}H]DPCPX. Levels of these receptors were increased from 389 ± 79 to 844 ± 250 fmol/mg protein by cisplatin (Fig. 1a). The equilibrium dissociation constants (K_d values) were 3.3 ± 1.2 nM and 5.1 ± 1.0 nM, respectively, for control and cisplatin-treated cells. A similar increase in A_1AR was obtained using ^{125}I-APNEA, an agonist radioligand for the A_1AR (Fig. 1, c-e). Western blotting studies performed on these membranes using a selective antibody for the A_1AR demonstrated increased levels of immunoreactivity (36–38 kDa band) in preparations derived from cisplatin-treated cells (Fig. 1b), which coincide with the size of the A_1AR by photoaffinity labeling. These data suggest that the increase in ^{125}I-APNEA binding probably represents an increase in total receptor number and not enhanced coupling to G proteins. In support of this, no change in the expression of G_i protein subunits was detected in cells treated with cisplatin for 24 hr (data not shown). The dose of cisplatin producing half maximal increase in radioligand binding (EC_{50}) was approximately 300 nM, whereas maximal increase in receptor levels was obtained with 2.5 μM cisplatin (Fig. 1c). With use of the latter concentration of cisplatin, optimal increases in receptors were obtained after 24 hr of treatment. Other chemotherapeutic agents known to generate free radicals were similarly effective in increasing the levels of A_1AR in these cells (Fig. 1d). The exception was transplatin, a platinum analog devoid of antitumor activity. Cells incubated with hydrogen peroxide (10 μM) for 24 hr also demonstrated increases in A_1AR levels, albeit to a lesser degree, suggesting a direct role of ROS in this process (Fig. 1d). Coincubation of cells with H_2O_2 and the antioxidant enzyme catalase blocked the increase in receptor expres-

sion. Administration of catalase alone led to reduction in receptor expression, supporting a role of ROS in the up-regulation of A_1AR. The increase in receptor level produced by cisplatin was attenuated after pretreatment with actinomycin D (4 μM) or cycloheximide (5 μg/ml) before administering cisplatin, suggesting a requirement for *de novo* synthesis of A_1AR in this process. Interestingly, although treatment with actinomycin D for 24 hr did not affect the basal expression of these receptors (110 ± 4% of control), administration of cycloheximide for the same period reduced receptor expression to 38 ± 1% of control, suggesting a fairly rapid rate of turnover of the A_1AR protein.

Chemotherapeutic agents such as cisplatin (Ravi *et al.*, 1995; Rybak *et al.*, 1995; Sasada *et al.*, 1996) and daunorubicin (Wang *et al.*, 1997) mediate their toxicities, in part, by generating ROS (Rybak *et al.*, 1995). In the case of daunorubicin, the generation of ROS presumably leads to activation of transcription factor NFκB (Beg and Baltimore, 1997). To test whether the increase in A_1AR induced by cisplatin was mediated through NFκB, the response to cisplatin was tested in cells pretreated with PDTC, an inhibitor of NFκB (Sun *et al.*, 1995). PDTC abrogated the increase in A_1AR expression induced by cisplatin (Fig. 1e). Similarly, inhibition of NFκB by dexamethasone (Mukaida *et al.*, 1994) also attenuated the response to cisplatin (Fig. 1e). PDTC was also effective in blocking the increase in A_1AR elicited by H_2O_2 (Fig. 1e), supporting the role of NFκB in the latter response. Additional experiments were performed by incubating cells with genistein, a different inhibitor of NFκB. This agent significantly reduced cisplatin-induced increase in A_1AR expression from 168 ± 15% to 128 ± 9% of control (three experiments). Administration of genistein alone had little effect on the expression of the A_1AR. The level of binding was 101 ± 3% of control. These experiments clearly underscore a role for NFκB in the regulation of A_1AR expression.

To determine whether exposure to cisplatin promotes activation of NFκB in DDT_1MF-2 cells, nuclear translocation of the p65 DNA binding subunit of NFκB was determined in Western blotting studies. Cisplatin increased the levels of p65 in the nucleus by 2- to 3-fold (Fig. 2a). Similar increases in the levels of c-Rel p75, a member of the NFκB family (Baeuerle, 1991; Baeuerle and Baltimore, 1996), were observed after cisplatin treatment (Fig. 2b). To test whether the increase in NFκB in the nucleus was associated with increased DNA binding activity, electrophoretic mobility shift assays were performed using an end-labeled NFκB DNA binding oligonucleotide (5′-ATGTGAGGGGACTTTC-CCAGGC-3′) (Sen and Baltimore, 1986). Retardation of two bands was observed (Fig. 2c), the appearance of which was

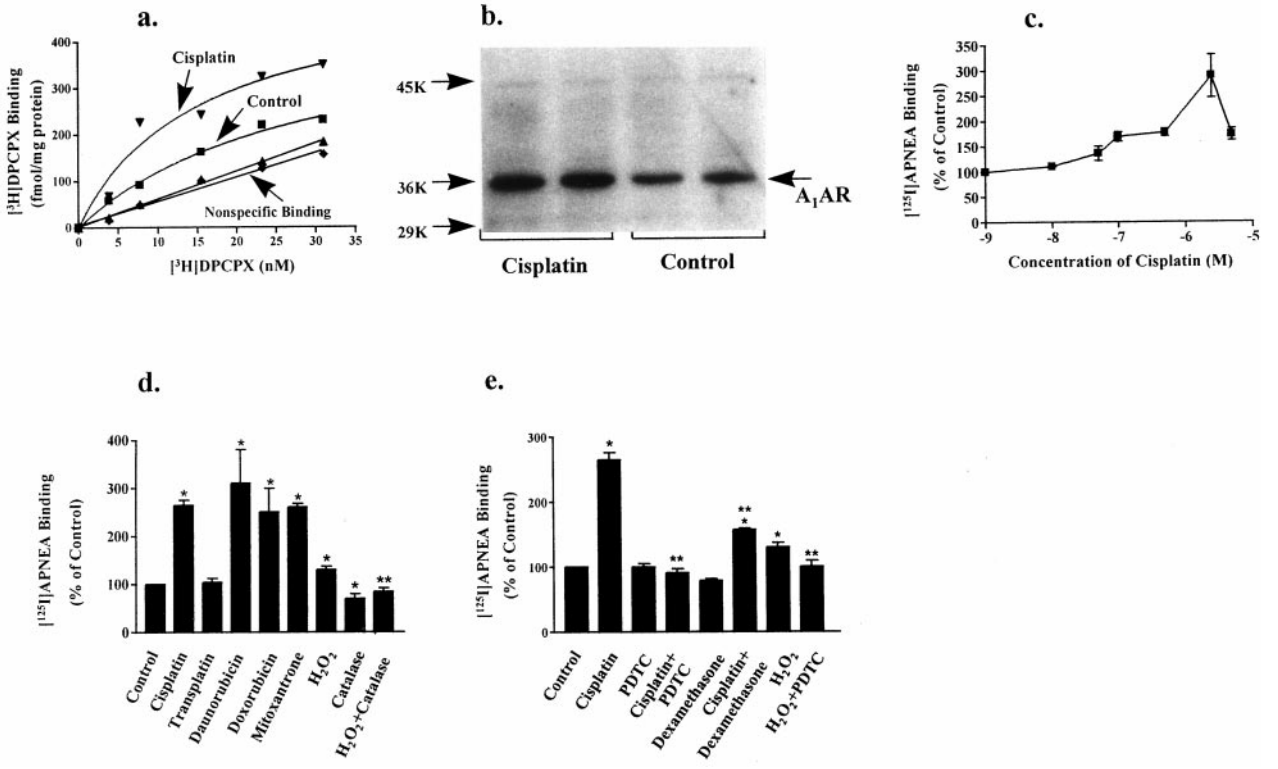

Fig. 1. Cisplatin for 24 hr induced up-regulation of A_1AR in DDT_1MF-2 cells. Cells were treated with cisplatin (2.5 μM) for 24 hr, after which crude plasma membranes were prepared for determining A_1AR expression using [^{3}H]DPCPX or ^{125}I-APNEA. a, Saturation curves showing increase in A_1AR expression in DDT_1MF-2 cells after cisplatin treatment. Saturation curves and receptor levels were analyzed by computer as described previously (Hancock *et al.*, 1979; De Leon *et al.*, 1982). b, Western blotting for the A_1AR using a polyclonal antisera at a titer of 1:1000. Molecular weight markers are indicated on the left. c, Dose-response effect of cisplatin obtained with cells treated with cisplatin for 24 hr. In this and subsequent experiments (d, e), a single, nonsaturating concentration of ^{125}I-APNEA (1 nM) was used. d, Up-regulation of A_1AR by different chemotherapeutic agents and H_2O_2. Cells were incubated with 1 μM of the chemotherapeutic agents or 10 μM H_2O_2 or 200 units/ml catalase for 24 hr. e, Inhibition of NFκB by PDTC, dexamethasone, or genistein. This treatment abrogated the stimulatory effect of cisplatin and H_2O_2 on A_1AR expression. Cells were treated with 200 μM PDTC, 100 nM dexamethasone, or 1 μM genistein. *, statistically significant difference from control; **, statistically significant difference from the cisplatin-treated group, using analysis of variance followed by *post hoc* analysis using Tukey's test. Experiments presented were repeated at least three times.

blocked by the addition of 30- and 10-fold excess (0.6 and 0.2 pmol, respectively) of cold κB probe (Fig. 2c, *lanes 8* and *9*). The addition of p65 antibody (1.0 and 0.5 μg/ml) to the reaction mixture (Fig. 2c, *lanes 10* and *11*, respectively) resulted in supershift of the upper band (Fig. 2c, *lane 11*), suggesting the presence of p65 in this complex. It is not clear why no supershifted band was detected in *lane 10*. Antibody against c-Rel (1 μg/ml) failed to significantly reduce DNA binding activity, possibly implying lower levels of this protein in the NFκB complexes. Administration of cisplatin to the DDT_1MF-2 cells increased DNA binding activities of both by approximately 4-fold (Fig. 2c, compare *lanes 2–4* with *lanes 5–7*). Similar electrophoretic mobility shift assays performed using a labeled oligonucleotide probe for the AP-1 transcription factor binding sequence indicate no change in the levels of these AP-1 factors after cisplatin treatment (Fig. 3). The intensities of the AP-1 labeled complexes were similar in the control versus cisplatin-treated cells (Fig. 3, compare *lanes 2–4* with *lanes 8–10*). Inhibition of the labeled probe could be detected with increasing concentrations of cold AP-1 oligonucleotide (Fig. 3, *lanes 5–7*). This suggests that cisplatin induction of A_1AR expression is mediated via NFκB and not by AP-1 transcription factors.

Analysis of the human A_1AR promoter sequence (Ren and Stiles, 1995) using Transfac (Quandt *et al.*, 1995; Wingender *et al.*, 1997) indicates the presence of consensus sequences for both NFκB and AP-1 transcription factors. A consensus sequence for NFκB (5′-GGAAGTCCC-3′) was detected 623 base pairs upstream of promoter A transcription start site in plasmid construct pBLPnif/PmtA (Ren and Stiles, 1995). An oligonucleotide probe constructed incorporating the sequence 5′-AGCAAGGGACTTCCGGAGGT-3′ was used as competitor for labeled κB probe in electrophoretic mobility shift assays. Results presented in Fig. 4a indicate inhibition of binding of the labeled probe with increasing concentrations (0.3–1.2 pmol) of A_1AR oligonucleotide (15- to 60-fold molar excess).

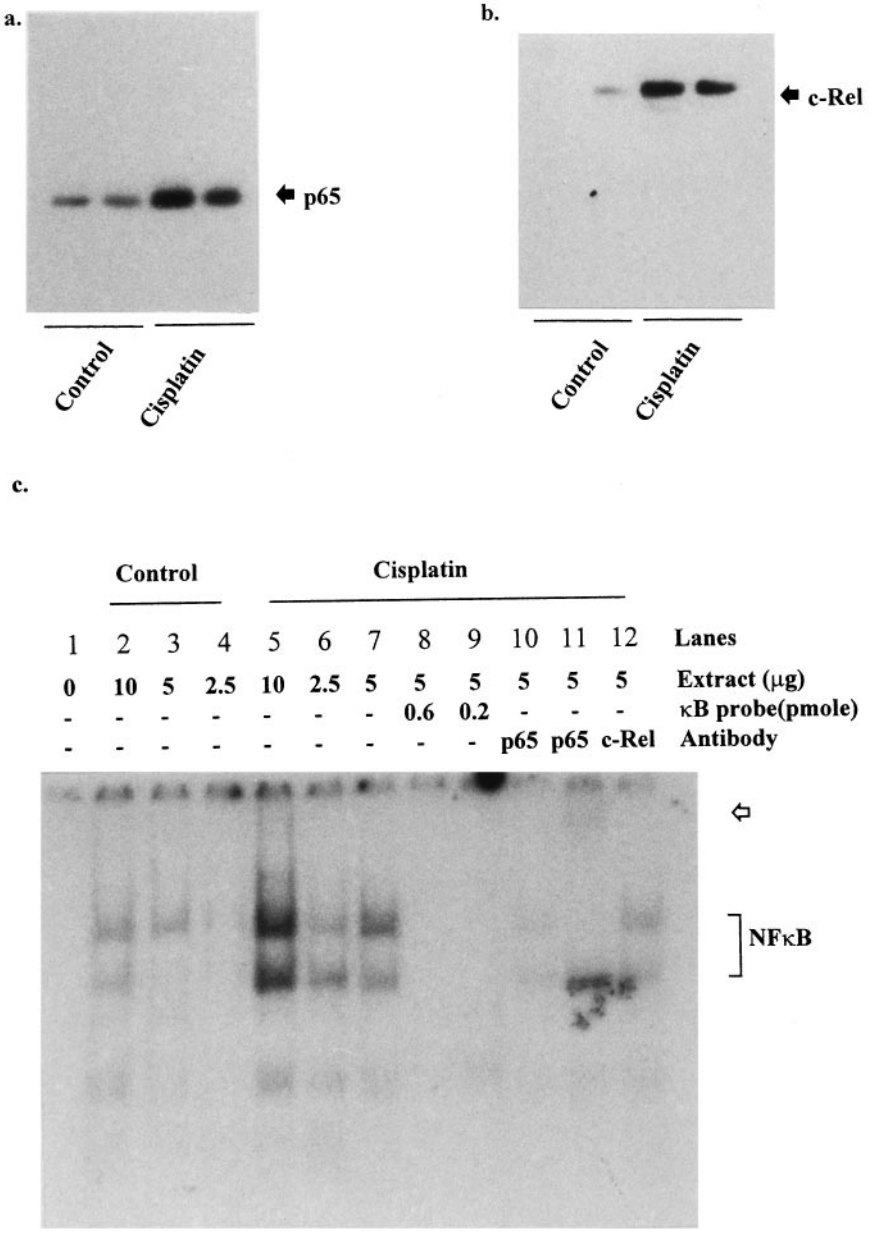

Fig. 2. Nuclear translocation of NFκB by cisplatin in DDT_1MF-2 cells. Cells were exposed to cisplatin (2.5 μM) for 2 hr and the nuclear translocation of NFκB complex was determined. Western blotting of p65 (a) and p75 (b) in the nucleus after exposure to cisplatin. Increase in NFκB (c) as detected in electrophoretic mobility shift assays. *Lanes 2–4* represent nuclear extracts from control cells, whereas *lanes 5–12* are extracts from cisplatin-treated cells. Two complexes, denoted NFκB, were detected. Note the increase in the binding of the κB probe in the cisplatin-treated cells (compare *lanes 2–4* with *lanes 5–7*). Binding of the labeled κB probe was inhibited by a 30- and 10-fold molar excess of cold κB probe (*lanes 8* and *9*). Samples were incubated with 1.0 μg/ml (*lane 10*) or 0.5 μg/ml (*lane 11*) of p65 antibody for supershift experiments. ◊, position of the supershifted band (*lane 11*).

Labeling of both the upper and lower bands were inhibit (Fig 4a, compare *lane 2* with *lanes 3–5*), whereas no change in intensity of the nonspecific band was detected. As shown before, unlabeled κB probe effectively competed with the labeled probe (Fig 4a, *lanes 6* and *7*) whereas no inhibition was observed using a 30-fold molar excess of unlabeled AP-1 oligonucleotide (Fig 4a, *lane 8*). To test whether the increase in nuclear translocation of NFκB could increase activity of the A_1AR gene promoter, DDT_1MF-2, cells were transiently transfected with pBLPnif/PmtA, which contains the firefly luciferase reporter gene driven by the A_1AR promoter (Ren and Stiles, 1995). Transfectional efficiency in each well was determined by cotransfecting a pCMV-CAT construct and assaying for CAT activities. Fig. 4b indicates a 2- to 3-fold increase in luciferase activity when these cells were treated with cisplatin for 24 hr. Because these experiments were performed with additional stress to the cells due to transfection, it is likely that this stress contributes to higher basal luciferase activity. To test whether the high basal activity was caused by ROS, culture media from control and cisplatin-treated cells were supplemented with either superoxide dismutase or catalase. Significant inhibition of luciferase activity was obtained in the presence of catalase but not superoxide dismutase, suggesting a role of H_2O_2 and not O_2^- in the regulation of A_1AR gene activity. Furthermore, inhibition of NFκB by PDTC led to significant reductions in the activity of the A_1AR promoter, supporting a role for NFκB in the maintenance of normal A_1AR expression under control conditions. When cisplatin-stimulated luciferase activity was compared with the basal activity defined by PDTC (200 μM), a 10- to 15-fold increase in luciferase activity was obtained (Fig. 4b). DDT_1MF-2 cells transfected with plasmid (pBLPnif/PmtB) containing promoter κB and a putative NFκB consensus sequence (5′-GGGGCTCCCC-3′) located 306 base pairs upstream of the second transcription start site (Ren and Stiles, 1995) did not exhibit any cisplatin-stimulated luciferase activity. These data suggest that while NFκB plays a significant role in regulating A_1AR gene activity, it is likely the consensus sequence upstream of promoter A that is important in this regulation.

If the increases in A_1AR obtained after cisplatin treatment were a compensatory response to increased oxidative stress, one would expect modulation of receptor activation to regulate the degree of oxidative stress the cell experiences. To test whether this increase in A_1AR confers protection in these cells, the levels of MDA, a marker of lipid peroxidation, were determined under different culture conditions (Ohkawa *et al.*, 1978). As shown in Fig. 5, cisplatin treatment for 24 hr led to an approximately 2-fold increase in MDA levels, which was reduced after activation of the A_1AR by 1 μM (*R*)-PIA. This protective effect of (*R*)-PIA was blocked after the addition of 1 μM of DPCPX, suggesting a cytoprotective role of the A_1AR in these cells. The reduction in MDA was also blocked

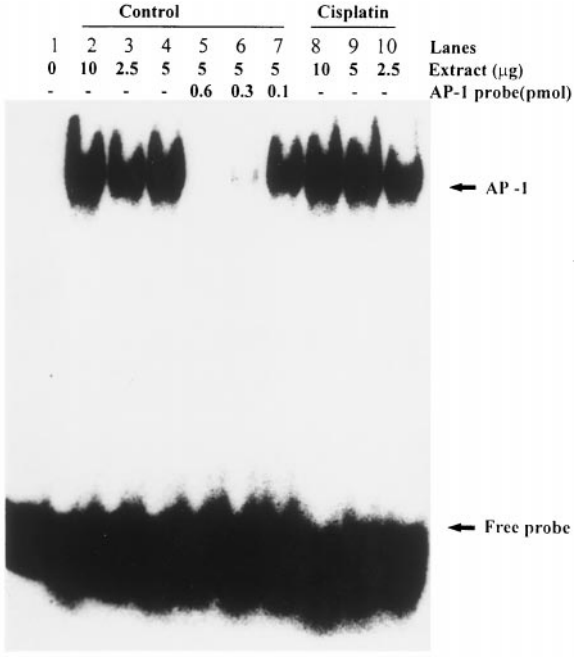

Fig. 3. Cisplatin treatment did not increase AP-1 binding activity in the nucleus. Cells were treated with cisplatin (2.5 μM) and nuclear extracts were prepared as described in Results and Discussion. Incubation of preparations from control and cisplatin-treated cells with labeled AP-1 probe indicates no difference in binding (compare *lanes 2–4* with *lanes 8–10*). Labeling of the labeled complex is inhibited in the presence of 30-, 15-, and 5-fold molar excess of cold AP-1 probe (*lanes 5–7*).

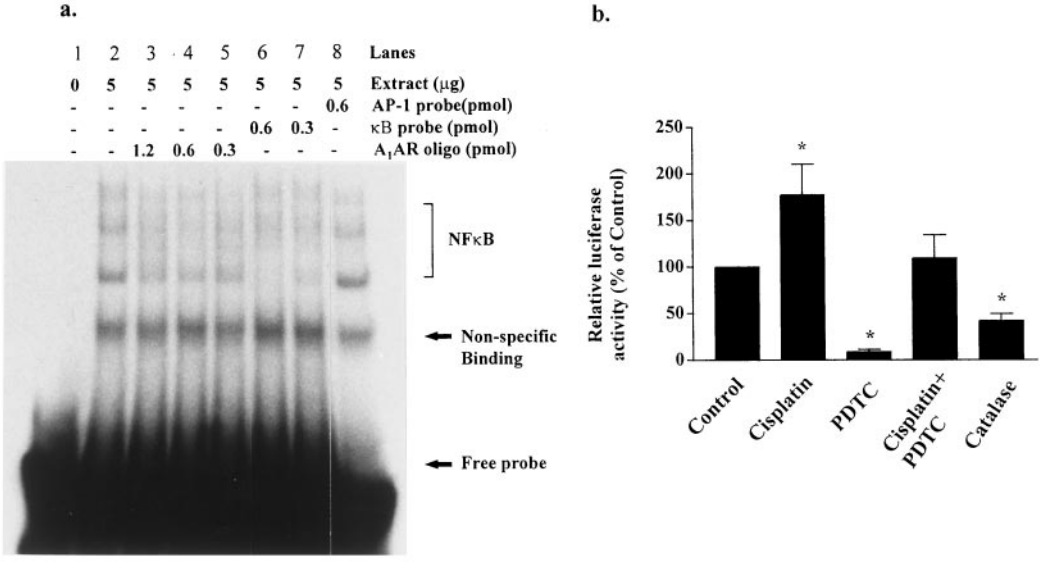

Fig. 4. The A_1AR promoter (promoter A) contains an NFκB regulatory site whose activity is induced by cisplatin. a, An A_1AR-derived κB oligonucleotide competing with the labeled probe for binding. Increasing concentrations of cold A_1AR oligonucleotide (*lanes 3–5*) reduced binding of both upper and lower NFκB bands. Inhibition of these two bands was also observed with cold κB probe (*lanes 6, 7*), but not with AP-1 probe (*lane 8*). b, Cisplatin-induced activation of luciferase activity. DDT_1MF-2 cells were transfected with plasmid (pBLPnif/PmtA) that contains the A_1AR promoter (promoter A) coupled to the firefly luciferase reporter gene (Ren and Stiles, 1995). Luciferase activity was determined in controls or in cells exposed to cisplatin for 24 hr in the absence or presence of PDTC (200 μM) or catalase (200 units/ml).

after incubation of the cells with 100 μM theophylline, a nonselective antagonist of the A_1AR.

Taken together, this study provides strong evidence for a direct role of NFκB in the expression of the A_1AR. Up-regulation of the A_1AR by this mechanism provides a means by which ischemic tissues can optimize the cytoprotective role of adenosine. Furthermore, the increase in A_1AR expression during ischemia may compensate for any down-regulation of this receptor (Lee *et al.*, 1986) resulting from a substantial rise in adenosine levels (Berne and Rubio, 1974). Down-regulation of the A_1AR during ischemia would limit the cytoprotective role of adenosine. These data suggest a paracrine role for ROS, regulating the expression of the A_1AR and providing cytoprotection to cells in the local environment. For the cancer cells, increase in the A_1AR may aid in the development of resistance to chemotherapeutic agents.

The present data support the notion that NFκB acts as a sensor of the oxidative status of the cell and regulates the expression of the A_1AR accordingly. As such, the extracellular environment can directly regulate the levels of this G protein-coupled receptor. Accordingly, one could titrate the expression of the A_1AR by altering the level of ROS in the extracellular environment. Such a close regulation of receptor expression could aid in cell survival during prolonged periods of exposure to ROS. Our data indicate that ROS and NFκB contribute significantly to A_1AR gene activity (at least for promoter A) because substantial decreases in activity were observed in the presence of catalase and after inhibition of this transcription factor.

Although cisplatin treatment led to significant changes in NFκB activity, there was surprisingly little change in AP-1 as assessed by electrophoretic mobility shift assays. Several AP-1 consensus sequences have been identified in the A_1AR promoter region (Ren and Stiles, 1995). Thus, it is possible that these sites are not important in ROS-mediated increases in A_1AR expression but may regulate receptor expression induced by other stresses. Alternatively, AP-1 sites may be important in maintaining a baseline level of A_1AR expression.

The administration of cisplatin is associated with significant oto- and nephrotoxicity in animals and humans (Rybak *et al.*, 1995). In rats and chinchillas, the administration of cisplatin resulted in significant elevation in A_1AR in the cochleas and kidneys (Ford *et al.*, 1997). Because the A_1AR mediates renal vascular constriction (Macias *et al.*, 1983), it is possible that the renal toxicity produced by cisplatin is mediated, in part, by up-regulation of this receptor subtype. Accordingly, blockade of these receptors with selective antagonists may alleviate some of the renal toxicity produced by this chemotherapeutic agent. Furthermore, activation of these A_1AR through round window application of selective agonists may alleviate the ototoxic effect of cisplatin in cancer patients.

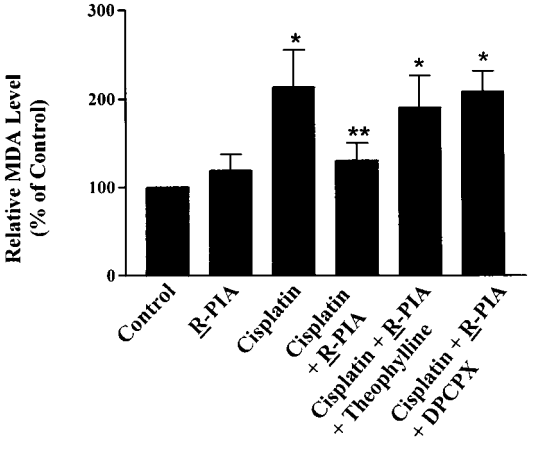

Fig. 5. Cytoprotective role of the increase in A_1AR induced by cisplatin. DDT$_1$MF-2 cells were treated with cisplatin (2.5 μM) for 24 hr, with or without the various drugs listed. The concentrations of (*R*)-PIA, DPCPX, and theophylline were 1 μM, 1 μM, and 100 μM, respectively. The results presented are the mean ± standard error of six independent experiments. Basal level of MDA in the control was 0.15 ± 0.05 nmole/mg protein. The data are presented as the mean ± standard error of three to five experiments. *, statistically significant difference ($p < 0.05$) from control. **, statistically significant difference from cisplatin-treated group.

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