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Chronic hypoxia up-regulates expression of adenosine A_1 receptors in DDT_1-MF2 cells

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Abstract

As the first step to understand how chronic hypoxia might regulate smooth muscle function in health and disease, we have employed an established immortalised cell model of smooth muscle, DDT_1-MF2 cells, to address the hypothesis that adenosine A_1 receptor density is modulated by O_2 availability. Maximal specific binding (B_{max}) of the selective adenosine A_1 receptor antagonist, [^{3}H]-DPCPX, to cell membranes increased 3.5-fold from 0.48 ± 0.02 pmol/mg to 1.7 ± 0.5 pmol/mg protein after 16 hr of hypoxia and this effect was not accompanied by any statistically significant changes in either binding affinity (0.84 ± 0.2 nM vs. 1.2 ± 0.3 nM) or Hill coefficient (1.1 ± 0.1 vs. 0.99 ± 0.03). Hypoxia-evoked increases in membrane receptor density were paralleled in intact DDT_1-MF2 cells. In addition, the increase in [^{3}H]-DPCPX binding to intact cells was inhibited by co-incubation during hypoxia with the translational inhibitor cycloheximide, the transcriptional blocker actinomycin D and the NFκB inhibitor sulphasalazine. Together, these data show that adenosine A_1 receptor density is modulated, at least in part, by O_2-dependent activation of the transcription factor NFκB and adds to the list of processes dynamically regulated by ambient oxygen availability. Since hypoxia is an initiating factor in acute renal failure, similar changes in transcription may account for up-regulation of adenosine A_1 receptors noted previously in the renal vasculature of rats with acute renal failure.

Keywords: Hypoxia; Adenosine A_1 receptors; Up-regulation; Nuclear factor κB; DDT_1-MF2 cells

1. Introduction

Adenosine is involved in the regulation of various aspects of renal function including sodium reabsorption, renal blood flow and glomerular filtration rate [1,2]. Moreover, adenosine has an important role in the pathogenesis of ARF since administration of adenosine antagonists, particularly selective adenosine A_1 receptor antagonists, has been shown to ameliorate acute renal dysfunction in animals induced by either myohaemoglobinuria [3–5], cisplatin [6], ischaemia [7] or hypoxia [8]. Furthermore, adenosine mediates, at least in part, the renal haemodynamic effects of nephrotoxic radiocontrast media since, in both animals [9] and man [10], the nonselective adenosine antagonist theophylline reduces the fall in glomerular filtration rate produced by contrast media injection. ARF produced by myohaemoglobinuria is associated with an increased density of renal adenosine A_1 receptors [11], particularly within glomeruli [12] where these receptors have been located on vascular smooth muscle [13]. Adenosine A_1 receptors mediate constriction of the renal afferent arteriole [1]. Consequently, up-regulation of these receptors may account for the enhanced renal vasoconstrictor response to adenosine noted in animals with ARF induced by myohaemoglobinuria, an effect that may contribute to the maintenance of ARF [14].

Tissue hypoxia results from a number of disease states including localised ischaemic episodes. Where studied at the cellular level, chronic hypoxic insult evokes dramatic remodelling of a variety of integral membrane proteins. For example, selective suppression of voltage-gated K^+ channel expression has been documented in pulmonary vascular smooth muscle cells in chronic hypoxia [15] whilst differential increases in L-type Ca^{2+} channel expression occur in

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Abbreviations: ARF, acute renal failure; [^{3}H]-8-cyclopentyl-1,3-dipropylxanthine, [^{3}H]-DPCPX.

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a model chemosensory cell line (phaeochromocytoma, PC12), independently of any changes in functional expression of N-type and P/Q-type Ca^{2+} channels [16,17]. In addition, another established model of chemosensing (the neuroepithelial body-derived H146 cell line) remodels its cellular response following chronic hypoxia by differential down-regulation of L-type Ca^{2+} channel functional expression whilst N-type channel expression is enhanced [18]. Furthermore, significant remodelling of cellular function by hypoxia is not limited to ion channel activity as PC12 cells demonstrate up-regulation of adenosine A_{2A} receptors following chronic depression of oxygen availability [19].

The observations that hypoxia and ischaemia can initiate ARF [7,8], that adenosine A_1 receptors produce constriction of the renal afferent arteriole [20] and that increased A_1 receptor density has been noted in glomeruli in ARF [12], has led us to test the hypothesis that a chronic hypoxic insult evokes transcription-driven augmentation of expression of adenosine A_1 receptors in smooth muscle cells. The present study was conducted using an established immortalised model of smooth muscle, the DDT_1-MF2 cell line.

2. Materials and methods

2.1. Materials

Reagents for cell culture were purchased from Gibco BRL except Dulbecco's modified Eagle's medium and foetal calf serum, as these were obtained from Sigma-Aldrich and Helena BioSciences, respectively. [^{3}H]-DPCPX (108.3 Ci/mmol) was purchased from Perkin-Elmer Life Sciences Inc. All other reagents were supplied by Sigma-Aldrich.

2.2. Cell culture

DDT_1-MF2 cells were purchased from the European Collection of Cell Cultures. Cells were grown in Dulbecco's modified Eagle's medium with 4.5 g/L glucose supplemented with: 10% foetal calf serum, 2 mM L-glutamine, 200 U/mL penicillin and 200 μg/mL streptomycin. Cells were grown at 37° in a humidified 5% CO_2:95% air atmosphere ($pO_2 \approx 143$ mmHg) and to about 80% confluence in 175 cm^2 flasks before they were used for experiments. All experiments were carried out with cells between passages 5 and 18. Cells exposed to hypoxia ($pO_2 \approx 18$ mmHg) were incubated in 2.5% O_2:5% CO_2 balanced with N_2 for up to 16 hr, as defined in the figure legends.

2.3. Binding of [^{3}H]-DPCPX to DDT_1-MF2 cell membranes

Cells were harvested from flasks by incubation with 10 mL of 10% trypsin-EDTA for 10 min at 37°. The resultant suspension was pelleted (800 *g* for 5 min) and washed twice with Kreb's phosphate buffer of composition (mM): 128 NaCl, 1.4 $MgCl_2$, 5.3 KCl, 10 Na_2HPO_4, pH 7.4 [21]. The cells were then resuspended in water to induce hypotonic lysis, homogenised with 15 strokes of a Potter homogeniser and centrifuged for 5 min at 800 *g*. The supernatant was centrifuged at 43,700 *g* for 25 min and the resultant pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.4) containing (mM): 10 $MgCl_2$, 1 EDTA plus 3 U/mL adenosine deaminase [21]. The suspension was incubated at 37° for 10 min and the membranes were repelleted by centrifugation at 43,700 *g* for 25 min. The resulting pellet was resuspended in Tris–HCl buffer containing 3 U/mL adenosine deaminase and stored at −80°.

Aliquots (0.1 mL; 20–30 μg protein) of cell membranes were added to plastic tubes containing 0.4 mL of [^{3}H]-DPCPX dissolved in Tris–HCl buffer. The final concentration of [^{3}H]-DPCPX ranged from 0.1 to 12 nM. Nonspecific binding was determined in the presence of 1 mM theophylline. Membranes and radioligand were incubated for 1.5 hr at 37° [21]. Following this, membranes were rapidly filtered through GF/C grade filters (Brandel). Filtration was performed with a Brandel cell harvester and filters were washed twice with about 5 mL of ice-cold phosphate-buffered saline, pH 7.4. Filters were then transferred to scintillation vials and incubated overnight in 12 mL of scintillation fluid before being counted for tritium.

2.4. Effect of hypoxia on the binding of [^{3}H]-DPCPX to whole cells

DDT_1-MF2 cells were incubated in hypoxic conditions ($pO_2 \approx 18$ mmHg) for 1, 2, 4, 8 and 16 hr. Cells were then harvested as described above. The resultant suspension was pelleted by centrifugation at 800 *g* for 5 min and washed twice with DMEM buffered with 20 mM HEPES (pH 7.4) containing 0.1% bovine serum albumin [22]. Finally, cells were resuspended at concentration of 1×10^6 cell/mL in HEPES buffered DMEM and the binding of 1 nM [^{3}H]-DPCPX was measured in the absence and presence of 1 mM theophylline, as described for the binding of this ligand to cell membranes.

2.5. Effect of actinomycin D, cycloheximide and sulphasalazine on the binding of [^{3}H]-DPCPX to whole cells

Cycloheximide and sulphasalazine were initially dissolved in dimethyl sulphoxide to give 1 mM solutions. Actinomycin D was dissolved in ethanol to give a concentration of 1 mg/mL. Aliquots (50–200 μL) were added to 25 cm^2 culture flasks containing 10 mL of culture medium and a layer of cells, about 80% confluent. Cells were then incubated for 4 hr in either hypoxic

($pO_2 \approx 18$ mmHg) or normoxic ($pO_2 \approx 143$ mmHg) conditions. Following this, cells were harvested, incubated with radioligand (1 nM) in the absence and presence of 1 mM theophylline and filtered, as previously described. Controls contained an appropriate volume of solvent.

2.6. Data analysis

Curves of specific binding were fitted by nonlinear regression analysis (Origin 5.0) to the equation: specific binding $= (B_{max} \times D)/(D + K_D)$ where B_{max} is the maximum specific binding, D is the concentration of [^{3}H]-DPCPX and K_D is the ligand dissociation constant. In addition, binding data were analysed by means of Scatchard and Hill plots. Data are given as mean ± SEM of N separate observations. Statistical significance was determined by Student's unpaired t-test with $P < 0.05$ taken as statistically significant.

3. Results

3.1. Effect of hypoxia on binding of [^{3}H]-DPCPX to DDT$_1$-MF2 cell membranes

The specific binding of [^{3}H]-DPCPX to membranes from DDT$_1$-MF2 cells grown in normoxia yielded a K_D of 0.84 ± 0.2 nM and a B_{max} of 0.48 ± 0.02 pmol/mg protein (N = 3, Fig. 1A). Fig. 1B and C show the Scatchard and Hill plots, respectively, for specific binding. The Scatchard plot could be fitted by a one-site model and the estimates of K_D and B_{max} were similar to those obtained for the binding isotherm shown in Fig. 1A. In addition, the Hill plot gave a slope of 1.1 ± 0.1 (N = 3) which was not significantly different from unity.

It is clear from Fig. 1A that incubation of DDT$_1$-MF2 cells for 16 hr under hypoxic conditions led to a substantial and significant increase in specific binding to cell membranes. Analysis of the binding isotherms gave a K_D of 1.2 ± 0.3 nM and a B_{max} of 1.7 ± 0.5 pmol/mg protein (N = 3). By comparison to the values obtained under normoxic conditions, there was a 3.5-fold increase in B_{max} but no statistically significant change in K_D. In addition, the Scatchard plot showed a parallel shift (Fig. 1B) and when displayed on a Hill plot, the data were superimposed on those obtained under normoxic conditions. The slope of the Hill plot for binding to membranes from cells exposed to 16 hr of hypoxia was 0.99 ± 0.03 (N = 3).

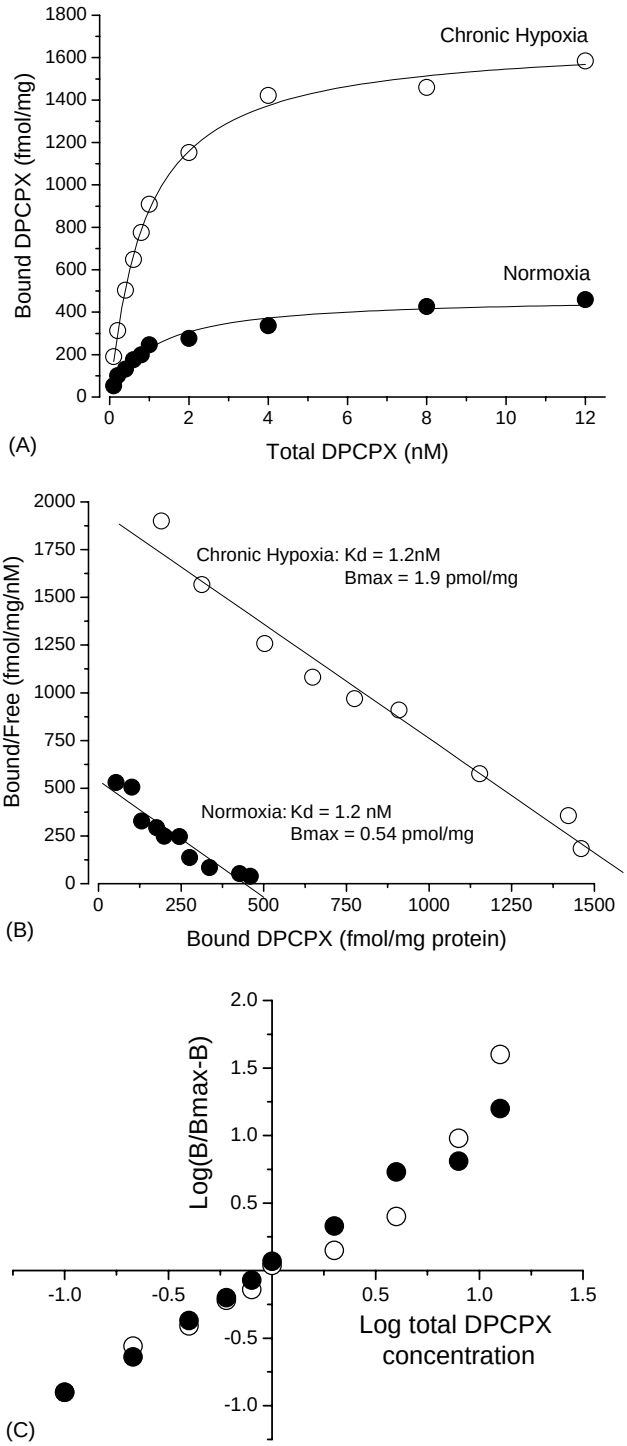

Fig. 1. Effect of chronic hypoxia on [^{3}H]-DPCPX binding to DTT$_1$-MF2 cell membranes. (A) [^{3}H]-DPCPX binding isotherms for membranes prepared from DTT$_1$-MF2 cells cultured for 16 hr in normoxia ($pO_2 \approx 143$ mmHg; filled circles) and hypoxia (≈18 mmHg; open circles). (B) Scatchard transformation of the mean data plotted in panel (A). (C) A Hill plot of mean data shown in panel (A). Each point represents the mean of three separate binding assays. The error bars have been omitted to aid clarity but were generally less than 5% of the mean.

3.2. Effect of hypoxia on the binding of [^{3}H]-DPCPX to whole DDT$_1$-MF2 cells

To investigate how the duration of hypoxia affects specific binding, the effect incubation time (1–16 hr) had on binding to whole cells was studied. Fig. 2A shows that 1–2 hr of hypoxia had no statistically significant effect on specific binding; but by contrast, 4–16 hr of hypoxia significantly ($P < 0.05$) increased specific binding

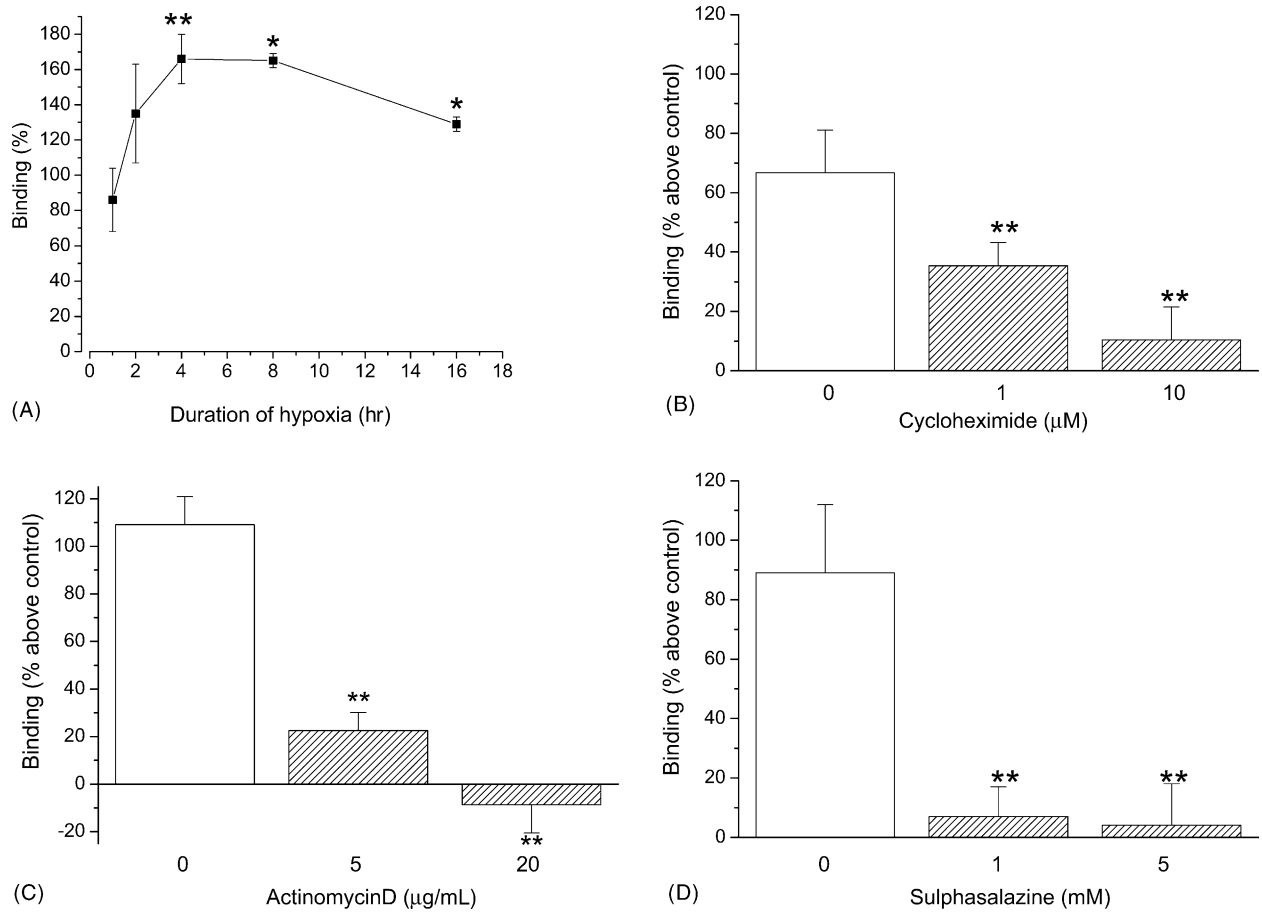

Fig. 2. Effect of chronic hypoxia on [^{3}H]-DPCPX binding to DTT$_1$-MF2 cells and its modulation by transcriptional and translational inhibitors. (A) Time course of increase in binding to intact cells of [^{3}H]-DPCPX during hypoxia ($pO_2 \approx 18$ mmHg). Binding to cells grown in normoxia ($pO_2 \approx 143$ mmHg) represents 100% at each time point. Effects of cycloheximide (B), actinomycin D (C) and sulphasalazine (D) on the increases in binding produced by 4 hr of hypoxia. For panels (B), (C) and (D), binding to cells grown in normoxia is defined as 0%. Values are mean ± SEM of three to five experiments. $^*P < 0.05$; $^{**}P < 0.01$ relative to control data in the absence of inhibitor (Student's *t*-test).

to whole cells. Four hours of hypoxia was chosen for experiments to investigate the effect of actinomycin D, cycloheximide and sulphasalazine on [^{3}H]-DPCPX binding since this time point represented the maximum enhancement of specific binding achieved under these conditions.

3.3. Effect of actinomycin D, cycloheximide and sulphasalazine on the binding of [^{3}H]-DPCPX to whole DDT$_1$-MF2 cells

Fig. 2B shows that inhibition of mRNA translation with cycloheximide (1 and 10 μM [23]) attenuated ($P < 0.01$) the hypoxia-induced increase in binding. Similarly, inhibition of DNA transcription with actinomycin D (5 and 20 μg/mL [24]) significantly ($P < 0.01$) blocked the increase in [^{3}H]-DPCPX specific binding induced by 4 hr of hypoxia (Fig. 2C). In addition, 1 and 5 mM sulphasalazine, a selective inhibitor of NF-κB activation at these concentrations [25,26], also prevented the increase in specific binding induced by a 4-hr hypoxic insult (Fig. 2D).

4. Discussion

The effect of hypoxia on adenosine A$_1$ receptor expression in DDT$_1$-MF2 cells is clear and dramatic. Between 2 and 16 hr of exposure to a partial pressure of O_2 as low as 18 mmHg evokes an increase in the binding of the selective A$_1$ receptor antagonist, [^{3}H]-DPCPX, to both isolated membranes and intact DDT$_1$-MF2 cells. This augmentation is due exclusively to increased specific binding (Fig. 1A and B) and occurs independently of modulation of kinetic parameters, as evidenced by the almost identical K_D values and Hill coefficients in both normoxia and chronic hypoxia. This conclusion is similar to that reached by Kobayashi *et al.* [19], albeit for the binding of the adenosine A$_{2A}$ selective agonist [^{3}H]-CGS21680 to PC12 cell membranes when these cells were subject to hypoxia (10% O_2 for 48 hr). That the increase in B_{max} is quantitatively larger in the membrane preparation than with whole cells almost certainly reflects the fact that the latter tonically release adenosine. Although the DDT$_1$-MF2 cells were washed twice following hypoxia, they were not treated with adenosine deaminase. In consequence, endogenous

adenosine may have occupied some A_1 receptors and partially masked the increase in receptor expression evoked by hypoxia [27].

We found that the enhancement of [^{3}H]-DPCPX binding is dependent on the duration of hypoxia. Ligand binding peaked after 4 hr of hypoxia and remained elevated for up to 16 hr of hypoxia. A similar time course has been noted for hypoxia-induced up-regulation of adenosine A_{2A} receptors in PC12 cells [19]. The present data provide evidence that augmented expression of A_1 receptors is mediated through activation of transcription and translation as selective suppression of transcription (using actinomycin D, Fig. 2C) or translation (using cycloheximide, Fig. 2B) significantly depress (at lower concentrations) or completely inhibit (at higher concentrations) the ability of hypoxia to increase the specific binding of [^{3}H]-DPCPX to cellular adenosine A_1 receptors. Furthermore, the primary mechanism of this hypoxia-evoked increase of receptor density is via activation and binding of the O_2-sensitive transcription factor NFκB since sulphasalazine, a selective inhibitor of NFκB activation [25,26], completely abrogated the action of 4 hr hypoxia (Fig. 2D). In this respect, it is interesting that Nie *et al.* [23] found that cisplatin and H_2O_2-induced increases in adenosine A_1 receptor expression in DDT_1-MF2 cells was also via activation of NFκB. It is not clear how NFκB is activated during hypoxia but hypoxia-induced proteolysis of the NFκB inhibitor IκBα may be involved [28]. In addition to NFκB, other transcription factors, for instance hypoxia-induced factor (HIF)-1, are also activated by hypoxia [29]. The present experiments do not exclude a role for such factors in hypoxia-induced up-regulation of A_1 receptors.

Up-regulation of adenosine A_1 receptors has been noted in glomeruli of rats with ARF induced by myohaemoglobinuria [12] whilst afferent arteriolar smooth muscle has been identified as the location for glomerular A_1 receptors [14]. These studies suggest that ARF is associated with increased expression of adenosine A_1 receptors in renal resistance vessels. Since ischaemia and hypoxia are common initiating factors in ARF, it is tempting to speculate that such events trigger increased expression of adenosine A_1 receptors in renal vascular smooth muscle in a manner similar to that noted with DDT_1-MF2 smooth muscle cells.

One question that arises from the current work is: What function does stress-induced up-regulation of adenosine A_1 receptors serve? On the one hand, increased expression of vascular adenosine A_1 receptors may be detrimental since A_1 receptors on renal vascular smooth muscle mediate constriction [1]. Accordingly, increased expression might exacerbate the renal vasoconstrictor effects of adenosine [14] and thereby contribute to the adverse haemodynamic changes that occur in ARF [30]. On the other hand, Lee and Emala [24] found that increased A_1 and A_{2A} receptor expression in immortalized human renal proximal tubule cells (HK-2), produced by chronic exposure to the nonselective adenosine receptor antagonist 8-phenyltheophylline, conferred significant protection against H_2O_2-induced oxidant injury. By contrast, down-regulation of these receptors enhanced the cytotoxicity of H_2O_2 [24]; thus up-regulation of adenosine A_1 receptors on tubule cells may be part of the cell defence mechanism to combat environmental stress.

In conclusion, this study has clearly demonstrated that hypoxia increases the expression adenosine A_1 receptors in DDT_1-MF2 cells by activation of the transcription factor NFκB. This novel finding provides a potential mechanism for the up-regulation of glomerular adenosine A_1 receptors noted in myohaemoglobinuric ARF [11,12] and the first evidence that O_2 availability may influence expression of these receptors in smooth muscle.

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