Activation of APE1/Ref-1 is dependent on reactive oxygen species generated after purinergic receptor stimulation by ATP

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ABSTRACT

Apurinic apyrimidinic endonuclease redox effector factor-1 (APE1/Ref-1) is involved both in the base excision repair (BER) of DNA lesions and in the eukaryotic transcriptional regulation. APE1/Ref-1 is regulated at both the transcriptional and post-translational levels, through control of subcellular localization and post-translational modification. In response to stress conditions, several cell types release ATP, which exerts stimulatory effects on eukaryotic cells via the purinergic receptors (P2) family. By using western blot and immunofluorescence analysis on a human tumour thyroid cell line (ARO), we demonstrate that purinergic stimulation by extracellular ATP induces quick cytoplasm to nucleus translocation of the protein at early times and its neosynthesis at later times. Continuous purinergic triggering by extracellular ATP released by ARO cells is responsible for the control of APE1/Ref-1 intracellular level. Interference with intracellular pathways activated by P2 triggering demonstrates that Ca^{2+} mobilization and intracellular reactive oxygen species (ROS) production are responsible for APE1/Ref-1 translocation. The APE1/Ref-1 activities on activator protein-1 (AP-1) DNA binding and DNA repair perfectly match its nuclear enrichment upon ATP stimulation. The biological relevance of our data is reinforced by the observation that APE1/Ref-1 stimulation by ATP protects ARO cells by H_2O_2-induced cell death. Our data provide new insights into the complex mechanisms regulating APE1/Ref-1 functions.

INTRODUCTION

Apurinic apyrimidinic endonuclease redox effector (APE1/Ref-1) is a protein involved both in the BER pathways of DNA lesions and in the regulation of gene expression as a redox co-activator of different transcription factors, such as Early growth response protein-1 (Egr-1), p53 and AP-1 (1). These activities are located into two functionally distinct domains: the N-terminus is principally devoted to the redox activity while the C-terminus exerts the enzymatic activity on the abasic sites of DNA (2).

APE1/Ref-1 is regulated at both the transcriptional and post-translational levels. In terms of transcriptional regulation, the effects of reactive oxygen species (ROS), such as H_2O_2, O_2^- and $OH^\bullet$, on APE1/Ref-1 induction have been the most intensively studied. Oxidative agents, such as H_2O_2, and ROS-generating injuries, such as UV-radiation, promote a transient APE1/Ref-1 induction, which correlates with an increase of its endonuclease and redox activities (1). The post-translational regulation of APE1/Ref-1 activities seems to reside into two non-mutually exclusive mechanisms, i.e. subcellular localization and post-translational modification degree. On one side, APE1/Ref-1 undergoes an active cytoplasm to nucleus translocation in different cell types upon ROS exposure (3,4). On the other side, phosphorylation and acetylation seem to play a role in determining the functional activity of the protein (1,5,6). However, neither molecular mechanisms responsible for the induction of APE1/Ref-1 translocation upon oxidative injury nor functional data regarding the 'in vivo' role played by

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post-translational modifications in controlling APE1/Ref-1 functions are clear (1). In particular, the 'primum movens' responsible for APE1/Ref-1 activation is completely unknown. Since APE1/Ref-1 is critical for the survival of mammals (7,8), it is important to dissect the signalling pathways responsible for its regulation.

Most reports have localized APE1/Ref-1 into the nucleus, but a growing body of evidence has shown that in some cell types, particularly those with high metabolic or proliferative rates, APE1/Ref-1 can be also cytoplasmic (1,3,4,9). Interestingly, this is a typical feature of some human tumours (1), such as the anaplastic thyroid carcinoma and the derived cell line (i.e. ARO cells) (3,10).

Generally, stimuli that promote APE1/Ref-1 expression are also able to promote its intracellular movement. The evidences reporting APE1/Ref-1 subcellular re-localization upon a stimulus have grown exponentially in the last few years (1). Most of these are cytoplasm-to-nucleus translocations. Different cellular conditions are able to perturb the APE1/Ref-1 intracellular localization (1); however, the way how eukaryotic cells regulate the re-localization of APE1/Ref-1 remains a completely open question.

In response to stress conditions or other stimuli, such as hypoxia, hyperoxia, mechanical stress, cAMP, hypotonic media, vasoactive agents and inflammation, several cell types release ATP via either a non-lytic (11,12) or a lytic mechanism from necrotic and apoptotic cells, reaching high local concentrations.

Extracellular nucleotides exert stimulatory effects on eukaryotic cells via the P2 family of membrane-bound receptors, which comprises P2Y (G-protein-coupled) and P2X (ligand-gated ion channel) receptors. Extracellular ATP improves the mitogenic action of several growth factors in a variety of cell types (13) through the control of transcriptional activators, such as AP-1 (14,15) and Egr-1 via stimulation of P2Y receptors (16). These effects are mainly played through the simultaneous increase of two important second messengers, Ca^{2+} ions and 1,2 diacylglycerol, a physiological activator of protein kinase C (PKC) (17). Interestingly, there are strong evidences demonstrating that the mitogenic action of P2Y triggering may be exerted by intracellular ROS generation (18).

Therefore, in the light of these observations, we tested the hypothesis of whether purinergic triggering may be able to affect APE1/Ref-1 functions by controlling its expression and subcellular localization.

MATERIALS AND METHODS

Cell culture and chemicals

ARO cells were cultured in RPMI medium containing 10% (w/v) fetal bovine serum (FBS), 5 mM glutamine and antibiotics. HOBIT cells were grown in DMEM F-12 (Invitrogen, Carlsbad, CA) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 100 U/ml penicillin and 10 μg/ml streptomycin sulfate. MG-63 osteoblast-like cells were grown in DMEM culture medium containing 10% (w/v) FCS, 100 U/ml penicillin and 10 μg/ml streptomycin sulfate. The cells were seeded in 100 mm dishes and grown at 37°C in a humidified atmosphere of 5% (v/v) CO_2. Both ATP and H_2O_2 treatments were performed on cells after 48 h of serum starvation and in medium w/o serum. Where indicated, protein neosynthesis was blocked by pre-treatment of the cells for 5 min with cycloheximide (CHX) (15 μg/ml) and for the whole duration of the experiment.

All the chemicals described below were from Sigma–Aldrich Co. (St Louis, MO) unless otherwise specified.

Preparation of total and nuclear cell extracts

Cell nuclear extracts were prepared as described previously (19). Briefly, 10^7 cells were washed once with phosphate-buffered saline (PBS) and resuspended in 100 μl of hypotonic lysis buffer A [10 mM HEPES, 10 mM KCl, 0.1 mM $MgCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 0.5 mM phenylmethylsulfonyl fluoride (PMSF), pH 7.9]. After 10 min, cells were homogenized by 10 strokes with a loose-fitting Dounce homogenizer. Nuclei were collected by centrifugation at $500 \times g$ at 4°C for 5 min in a microcentrifuge. The supernatant was considered as the cytoplasmic fraction. Nuclei were then washed three times with the same volumes of buffer A in order to minimize cytoplasmic contamination. Nuclear proteins were extracted with 100 μl of buffer B (10 mM HEPES, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.1 mM EDTA, 2 μg/ml leupeptin, 2 μg/ml pepstatin and 0.5 mM PMSF, pH 7.9). After incubating for 30 min at 4°C, samples were centrifuged at $12\,000 \times g$ for 20 min at 4°C. Nuclear extracts were then analysed for protein content (20) and stored at $-80°C$ in aliquots.

Western blot analysis

Aliquots containing 5 μg of both nuclear and cytoplasmic extracts, obtained from ARO cells incubated under different conditions, were electrophoresed onto a 12% SDS–PAGE. Proteins were then transferred to nitrocellulose membranes (Schleicher & Schuell, Keene, NH). Membranes were saturated by incubation, at 4°C overnight, with 5% non-fat dry milk in PBS/0.1% Tween-20 and then incubated with the polyclonal anti-APE1/Ref-1 antibody (21) for 3 h. After three washes with PBS/0.1% Tween-20, membranes were incubated with an anti-rabbit immunoglobulin coupled to peroxidase (Sigma, St Louis, MO). After 60 min of incubation at room temperature, the membranes were washed three times with PBS/0.1% Tween-20 and the blots were developed using enhanced chemiluminescence procedure (Amersham Pharmacia Biotech, Milan, Italy). Normalizations were performed with the polyclonal anti-actin antibody (Sigma, St Louis, MO). Blots were quantified by using a Gel Doc 2000 videodensitometer (Bio-Rad, Hercules, CA).

For western blot analysis of P2 receptors, 10^6 ARO cells were lysed in 100 μl of lysis buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM DTT, 0.5 mM PMSF, 0.1 mM NaF, 2 μg/ml leupeptin and 2 μg/ml pepstatin in PBS. After incubation for 30 min on ice, cellular extracts were centrifuged at $13\,000\,g$ for 15 min at 4°C and the supernatant was collected. Then, 50 μg of extracts were separated onto a 10% SDS–PAGE, blotted onto nitrocellulose membranes and assayed for the presence of $P2Y_1$ and $P2Y_2$ proteins by using specific polyclonal antibodies (Alomone Labs., Jerusalem, Israel). To assay for the specificity of the recognized bands, competition experiments, with the specific peptides of the two receptors, were performed by pre-incubating

each antibody for 30 min at room temperature with each specific peptide according to manufacturer's instructions before probing the membranes. Membrane blocking and bands detection were performed as described above.

Immunofluorescence and confocal microscopy

Cells were fixed for 20 min with 4% paraformaldehyde in PBS, treated for 5 min with 0.1% Triton X-100 in PBS. Cells were then incubated for 30 min at 37°C with 0.1 mg/ml RNase in PBS. Unspecific binding of the antibodies was blocked for 20 min with 1% FBS in PBS. The primary monoclonal antibody anti-APE1/Ref-1 (22) was incubated for 30 min at room temperature. Fluoresceinated secondary antibody was used to reveal the primary antibody.

After immunofluorescence treatment, nuclei were stained by 3 min incubation in 1 μg/ml solution of propidium iodide in PBS. Inhibition of protein synthesis was carried out by adding CHX 5 min before and during the stimulation time. Immunofluorescent images were collected using a confocal microscope (Leica DM IRB/E, Wetzlar, Germany).

Determination of AP endonuclease activity

The determination of AP endonuclease activity was performed using an oligonucleotide cleavage assay as described previously (6). Cell extracts were incubated with a $5'$-^{32}P-end-labelled 26mer oligonucleotide containing a single tetrahydrofuranyl (THF) artificial AP site at position 14, which, in the presence of AP endonuclease activity, is cleaved to a 14mer. The abasic analogue is resistant to cleavage by $3'$-acting AP lyase activity, which is generally possessed by DNA glycosylase/AP lyases. Therefore, this assay is specific for APE1/Ref-1 activity in cells. Reaction mixtures (20 μl) containing cell extracts of interest, 2.5 pmol of $5'$-^{32}P-end-labelled, double-stranded THF oligonucleotide, 50 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, 1 μg/ml BSA and 0.05% Triton X-100 (pH 7.5) were allowed to proceed for 15 min in a 37°C water bath. Reactions were halted by adding 10 μl of 96% formamide, 10 mM EDTA, xylene cyanol and bromophenol blue as dyes. AP assay products (5 μl) were separated on a 20% polyacrylamide gel containing 7 M urea. Gels were wrapped in saran wrap and exposed to film for autoradiography. The amount of 14mer–26mer was determined by scanning the exposed film into Gel Doc scanner (BioRad, Milan, Italy).

Electrophoretic mobility shift assay (EMSA) analysis of AP-1 DNA-binding activity

Double-stranded oligodeoxynucleotides, labelled at the $5'$ end with ^{32}P, were used as probes in gel retardation assays. The specific AP-1 consensus binding site, here named *AP-1 BS* oligonucleotide, is a 21mer whose upper strand is $5'$-CGCTTGATGAGTCAGCCGGAA-$3'$. The mutant AP-1 binding site that was used for competitions, here named *AP-1 MS* oligonucleotide, is a 21mer whose upper strand is $5'$-CGCTTGATGACCCAGCCGGAA-$3'$ (underlined the mutagenized bases). Both oligonucleotides were from Geneka Biotechnology Inc. (Montreal, Canada). The gel retardation assay was performed by incubating protein and DNA in a buffer containing 20 mM Tris–HCl, pH 7.6, 75 mM KCl, 0.25 μg/ml BSA with calf thymus DNA (25 μg/ml) and

10% glycerol for 30 min at room temperature. Protein-bound DNA and free DNA were separated on a native 5% polyacrylamide gel run in 0.5xTBE for 1.5 h at 4°C. The gel was dried and then exposed to an X-ray film at −80°C.

Recombinant APE1/Ref-1 expression and purification

Recombinant APE1/Ref-1 protein (rAPE1/Ref-1) was obtained as an hexahistidine-tag fusion protein from over-expression in *Escherichia coli* and then purified by nickel-chelate chromatography (23) from bacterial extracts and treated as described previously (2). Sample concentrations were determined by Bradford colorimetric assay (20). The purified proteins gave a single band on an overloaded SDS–PAGE. Fractions containing purified proteins were stored at −80°C. High-performance liquid chromatography analysis and mass spectrometry characterization confirmed the quality of the purified protein.

Cell viability assay

An MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay was used for the cell viability assay (24). After incubation with H_2O_2 for 1 h, a 1/10 volume of MTT solution (4 mg/ml in PBS) was added and incubated for 4 h under 10% CO_2/90% air at 37°C. Then, the supernatant was aspirated and an equal volume of dimethyl sulfoxide was added to the cells; the MTT formazan was dissolved by pipetting. The absorbance was measured on an enzyme-linked immunosorbent assay plate reader (EL808 Ultra Microplate Reader BIO-TEK Instruments, Inc., Winooski, VT) with test and reference wavelength of 570 and 630 nm, respectively.

Determination of intracellular ROS levels

Intracellular ROS levels were monitored by using the fluorescent dye $2',7'$-dichlorodihydrofluorescein diacetate (H_2DCFDA) (Molecular Probes, Eugene, OR), which is a non-polar compound converted into a non-fluorescent polar derivative (H_2DCF) by cellular esterases after incorporation into cells. H_2DCF is membrane impermeable and is oxidized rapidly to the highly fluorescent $2',7'$-dichlorofluorescein (DCF) in the presence of intracellular ROS (25). The day before the experiments, ARO cells were seeded at a density of 40 000 cells/cm^2. In the day of the experiment, after removing the medium with serum, the cells were washed in the plates with PBS and then incubated in 5% CO_2/95% air at 37°C with the ATP stimulus. Then, the H_2DCFDA (10 μM) was added 30 min before finishing cellular treatment. The cells were washed twice with PBS, harvested with trypsin and centrifuged at 800 *g* for 5 min and finally washed twice in PBS. A spectrofluorometer (LS-50B Perkin Elmer, Wellesley, MA) was used to measure the fluorescence intensity with the excitation and emission wavelengths at 505 and 530 nm, respectively.

RT–PCR analysis

Total RNA was purified from ARO or MG-63 cells using the SV Total RNA Isolation System from Promega (WI, USA) according to the manufacturer's protocol. RT–PCR was performed with 1 μg of total RNA as template. $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and control GAPDH mRNAs were reverse

transcribed using a 20mer oligo(dT) and amplified using the specific primers: P2Y$_1$ forward, 5′-TGTGGTGTACCCCC-TCAAGTCCC-3′ and P2Y$_1$ reverse, 5′-ATCCGTAACAGC-CCAGAATCAGCA-3′ (amplified of 259 bp) (14); P2Y$_2$ forward, 5′-CCAGGCCCCCGTGCTCTACTTTG-3′ and P2Y$_2$ reverse, 5′-CATGTTGATGGCGTTGAGGGTGTG-3′ (amplified of 362 bp) (14); P2Y$_4$ forward, 5′-CAGCAA-CAAAGGGACCAC-3′ and P2Y$_4$ reverse, 5′-GCTTGCCAC-CACCACAGA-3′ (amplified of 449 bp); P2Y$_6$ forward, 5′-ACAGGCATCCAGCGTAAC-3′ and P2Y$_6$ reverse, 5′-CGGACACAATGGCAAATA-3′ (amplified of 530 bp) (26); GAPDH forward 5′-TCTAGACGCCAGGTCAGGTCCACC-3 and GAPDH reverse 5′-CCACCCATGGCAAATTCCAT-GGCA-3′ (amplified of 598 bp) (26). The amplified products were resolved on a 1.5% agarose gel.

Statistical analysis

Statistical analysis was performed using the Microsoft excel data analysis program for Student's *t*-test analysis or the statistical analysis program for ANOVA with the Scheffè multiple comparison test. $P < 0.05$ was considered statistically significant.

RESULTS

ATP treatment modulates APE1/Ref-1 expression and trafficking

The ARO cell line is ideal for the study of APE1/Ref-1 subcellular localization as these cells present a huge amount of cytoplasmic APE1/Ref-1 (3,10). This allows monitoring and characterizing the APE1/Ref-1 cytoplasm to nucleus translocation at early times upon a stimulus. In order to follow the APE1/Ref-1 trafficking upon ATP stimulation, we performed western blot analysis after the separation of nuclear and cytoplasmic compartments. The time course of ATP stimulation demonstrates that the amount of APE1/Ref-1 in the nuclear compartment increases following 10 min of ATP (100 μM) stimulation and this increase persists until 60 min of stimulation (Figure 1A). On the contrary, we found a strong decrease of APE1/Ref-1 in the cytoplasmic fraction upon 10 min of ATP stimulation, followed by a new increase upon 30 min of ATP stimulation (Figure 1A). Indirect immunofluorescence experiments and confocal analysis (Figure 1B) were in agreement with the western blot data. These data suggest that ATP stimulation induced at least two events: first a nuclear translocation of APE1/Ref-1 then a neosynthesis of
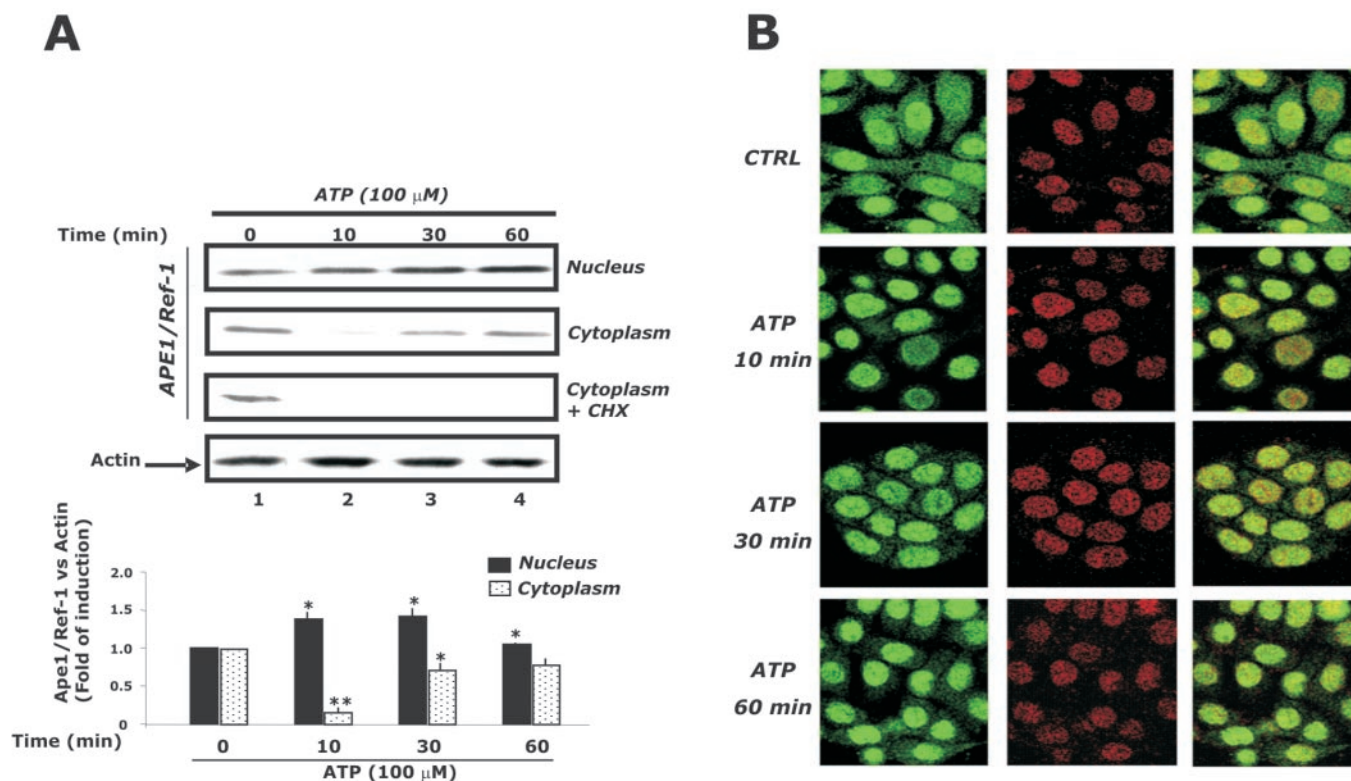

Figure 1. ATP affects both APE1/Ref-1 localization and expression. (**A**) ARO cells were cultured in serum-free medium for 48 h and then stimulated with ATP for the indicated times. Representative western blot analysis, performed as described in Materials and Methods, of APE1/Ref-1 expression levels of nuclear and cytoplasmic extracts of ARO cells stimulated for the indicated times with 100 μM ATP in the absence or in the presence of the protein synthesis inhibitor CHX (15 μg/ml) 5 min before and during the ATP treatment, as indicated. Actin was always measured, as loading control. Lower panel, values obtained from densitometric analysis of three independent western blot experiments were reported as histograms. Columns and bars indicate the means ± SD from three independent experiments. Black bars represent the nuclear levels of APE1/Ref-1 and the white dotted bars represent the cytoplasmic APE1/Ref-1 protein levels obtained from densitometric analysis of three independent experiments, normalized versus actin (*, $p \leqslant 0.05$; **, $p < 0.001$ compared with controls). (**B**) ARO cells were cultured in serum-free medium for 48 h and then stimulated with ATP for the indicated times. Cells were then fixed and stained for APE1/Ref-1 (green). Nuclei were visualized by propidium iodide counterstaining (red). Note that APE1/Ref-1 is mainly nuclear upon 10 min of stimulation.

APE1/Ref-1 protein at later times of stimulation. This was confirmed by inhibiting the protein synthesis apparatus with 15 µg/ml CHX, 5 min before and during the whole duration of the ATP stimulation time. The western blot analysis of the cytoplasmic fraction showed that APE1/Ref-1 was undetectable at each time of ATP stimulation in the presence of CHX (Figure 1A), suggesting that a later event of ATP stimulation is the induction of APE1/Ref-1 protein neosynthesis.

As for the trafficking of APE1/Ref-1, we treated the cells with CHX both in the presence and in the absence of ATP stimulation; then, we followed APE1/Ref-1 localization by western blot and confocal analysis. We found that following ATP stimulation the amount of nuclear APE1/Ref-1 increased, while it was undetectable in the cytoplasm (Figure 2A and B). An opposite situation was observed following treatment with CHX in the absence of ATP treatment. We found that 60 min of CHX treatment produced a decrease of APE1/Ref-1 in the nucleus and an increase in the cytoplasm where most of the protein is localized (Figure 2A and B). We hypothesized that APE1/Ref-1 redistribution represents the turn over of the protein, which becomes cytoplasmic and is further degraded. To test this hypothesis, we analysed the half-life of APE1/Ref-1 both in the presence and in the absence of ATP by treating the cells with CHX for different times. By western blot analysis of total cellular extracts, we found that ATP stimulation did not affect APE1/Ref-1 turn over (half-life), since the amount of this protein did not change significantly within 6 h of CHX treatment both in the presence and in the absence of ATP (data not shown). All these data demonstrated that ATP stimulation induced both APE1/Ref-1 translocation and its maintenance in the nucleus.

In order to assay for the sensitivity of APE1/Ref-1 nuclear translocation upon ATP stimulation, dose–response experiments were carried out at 10 min of ATP treatment (Figure 2C, lanes 1–4). Stimulatory effects were clearly detectable at doses of ATP as low as 10 and 1 µM, thus emphasizing the biological relevance of this phenomenon.

Purinergic receptors (P1 and P2) are natural candidates as targets for ATP stimulation. To test whether ATP activates P2-type receptors, ARO cells were pre-treated with P2 antagonist (100 µM suramin) 30 min before the addition of 100 µM ATP (27). Pre-treatment completely blocked the APE1/Ref-1 cytoplasm to nucleus translocation (Figure 2C, lanes 5 and 6), underlining the role of cell-surface P2 purinergic receptors in the extracellular ATP-mediated activation of APE1/Ref-1 in ARO cells.

Adenosine is the main agonist of P1 receptors (27) and it may be present in the medium as a by-product of ATP hydrolysis. Therefore, we performed experiments with the non-hydrolysable form of ATP (i.e. ATP-γS) and with adenosine. Data reported in Figure 2C (lane 7) clearly show that exposure of ARO cells to 100 µM ATP-γS promotes active APE1/Ref-1 nuclear translocation. On the contrary, adenosine treatment did not exert any effect in determining APE1/Ref-1 translocation definitely excluding any role for P1 receptors (lane 8).

To infer the class of P2 receptors (P2X or P2Y) that may be responsible for the ATP-induced APE1/Ref-1 nuclear translocation, we performed a series of experiments with P2Y specific agonists, UTP and ADP (27), being ATP a non-selective agonist of both P2X and P2Y receptors. ARO cells were treated with 100 µM UTP or with 100 µM ADP

for 10 min and APE1/Ref-1 amounts were assayed by western blot analysis performed on nuclear and cytoplasmic extracts (Figure 2D, lanes 3 and 4). Both UTP and ADP clearly exert a stimulatory effect, comparable with that obtained with ATP treatment (Figure 2D, lane 2), thus suggesting that P2Y receptors are mainly involved in the ATP-induced APE1/Ref-1 nuclear translocation. Moreover, the specificity of UTP for $P2Y_2$ receptors (27,28) is suggestive for a major involvement of $P2Y_2$ over $P2Y_1$ receptors in mediating the effect of ATP. To strengthen the role of P2Y receptors and to definitely exclude the involvement of P2X receptor class, experiments with the specific agonist of P2Y receptors (i.e. 2MeSADP) and with the specific agonist of P2X receptors (i.e. αβMeATP) were performed (Figure 2D, lanes 5 and 6). While the former promoted an active APE1/Ref-1 nuclear translocation, the latter was completely unable.
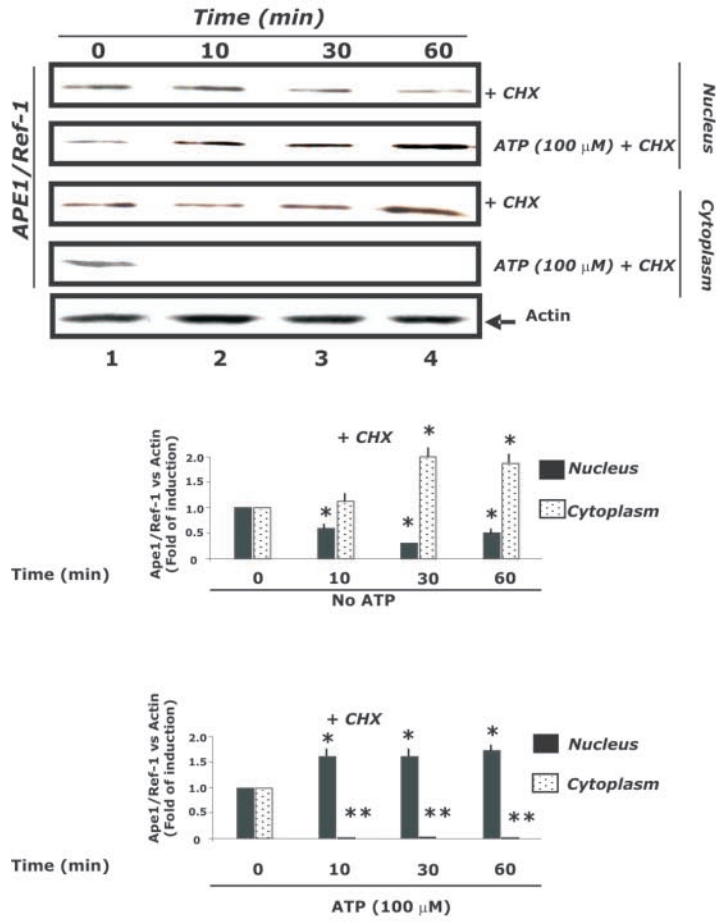
Further experiments were carried out to discriminate the different subclasses of P2Y purinoreceptors. Unlike $P2Y_1$ and $P2Y_1$-like receptors, $P2Y_2$ and $P2Y_4$ receptors are activated by both ATP and UTP and only weakly by ADP and other nucleoside diphosphates. It is known that $P2Y_6$ receptor is activated by UDP that, in our experimental conditions, can be present as a contaminant. To exclude the involvement of $P2Y_4$ and $P2Y_6$ in our system, we used RT–PCR to assay the expression of these receptors by ARO cells (Figure 2E, left). Indeed, while $P2Y_1$ and $P2Y_2$ are expressed, $P2Y_4$ and $P2Y_6$ are not, thus excluding their involvement in the APE1/Ref-1 activation. As a positive control for $P2Y_4$ and $P2Y_6$ amplifications, the cDNA of MG-63 cells expressing these two purinoreceptors was used as template. The signal obtained with primers specific for GAPDH was similar in all reactions, confirming the integrity and amount of cDNA in each sample. Western blot analysis, performed with specific antibodies (Figure 2E, right), confirmed the expression of both $P2Y_1$ and $P2Y_2$ receptors as proteins in the ARO cell line. Bands of ∼47 kDa in the case of $P2Y_1$ and of ∼50 kDa in the case of $P2Y_2$, which were efficiently competed by pre-incubation of the $P2Y_1$ and $P2Y_2$ antibodies with each specific peptide (Figure 2E, lane 3), were detected.

The data obtained with ARO cells claim for a major involvement of $P2Y_1$ and $P2Y_2$ receptors in mediating ATP-induced APE1/Ref-1 nuclear translocation. However, we cannot exclude at present the possible involvement of other P2Y receptors in other cell systems. Similar data were obtained in other cell systems, i.e. HOBIT osteoblast-like cells and PC-12 neuronal cells (data not shown), reinforcing the general physiological importance of this kind of activator signalling.
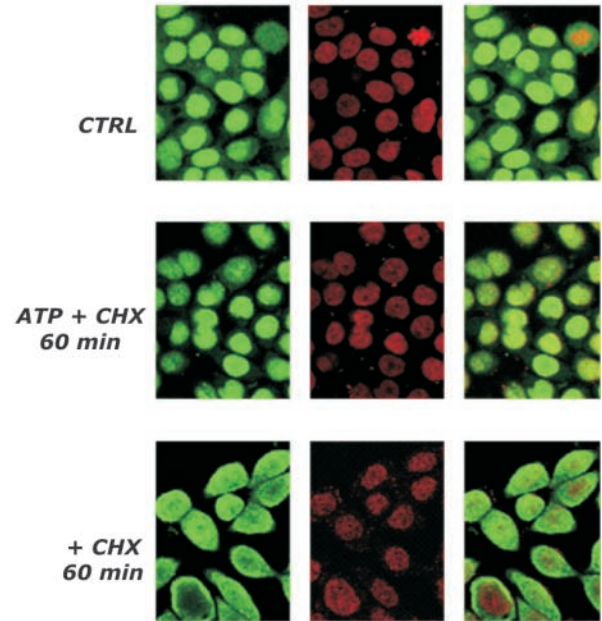
Continuous purinergic triggering is responsible for the control of the APE1/Ref-1 subcellular levels

In order to test whether basal ATP release is associated to the control of the subcellular localization and expression of APE1/Ref-1 protein, we inhibited the possible effects due to autocrine triggering of P2 receptors by treating the cells with the ATP/ADPase apyrase and with the P2 receptor antagonist suramin for extended times (Figure 2F). Treatment of ARO cells with 5 U/ml of apyrase (for 24 and 48 h) significantly reduced both nuclear and cytoplasmic amounts of APE1/Ref-1 protein. Similar data were obtained by treatment with 100 µM suramin. These data unequivocally demonstrate
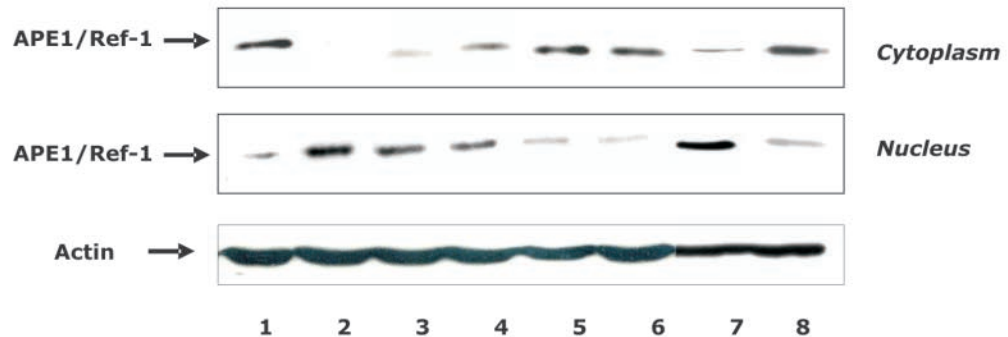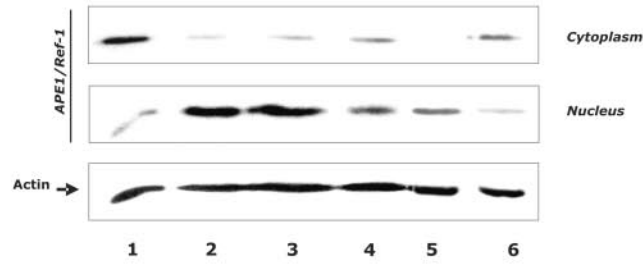
A

B

C

D

CHX (15 μg/ml)	+	+	+	+	+	+
ATP (100 μM), 10 min	−	+	−	−	−	−
UTP (100 μM), 10 min	−	−	+	−	−	−
ADP (100 μM), 10 min	−	−	−	+	−	−
2MeS-ADP (100 μM), 10 min	−	−	−	−	+	−
αβMeATP (100 μM), 10 min	−	−	−	−	−	+

APE1/Ref-1

Cytoplasm

Nucleus

Actin →

1   2   3   4   5   6

E

ARO

P2Y₁   P2Y₂   No RT   No RT

362 bp
259 bp

GAPDH   598 bp

1   2   3   4

WB: α-P2Y₁

WB: α-P2Y₂

1   2   3

ARO   MG-63   ARO No RT   MG-63 No RT

P2Y₄   449 bp

P2Y₆   530 bp

GAPDH   598 bp

1   2   3   4

F

Apyrase (5 U/ml)	−	−	24 h	−	−	48 h
Suramin (100 μM)	−	24 h	−	−	48 h	−

APE1/Ref-1

Cytoplasm

Nucleus

Actin →

1   2   3   4   5   6

that continuous purinergic triggering by extracellular released ATP is involved in controlling APE1/Ref-1 subcellular levels. The downregulation of APE1/Ref-1 nuclear and cytoplasmic levels were not due to degradative pathways induced by alternative triggering by accumulation of AMP or adenosine due to the apyrase activity, since neither AMP (data not shown) nor adenosine (shown in Figure 2C, lane 8) played any effect in controlling APE1/Ref-1 subcellular localization. Quantification of the amounts of APE1/Ref-1 protein levels (in terms of nuclear/cytoplasmic ratio, $R_{n/c}$) upon suramin and apyrase treatments evidenced that, in association to a reduction in the APE1/Ref-1 expression levels, the nuclear compartment resulted emptied in a time-dependent manner ($R_{n/c}$ control: 1.2; $R_{n/c}$ apyrase 24 h: $R_{n/c}$ 0.9; $R_{n/c}$ apyrase 24 h: 0.7; $R_{n/c}$ suramin 24 h: 0.45; $R_{n/c}$ suramin 48 h: 0.3). These data parallel those obtained by CHX treatment confirming the hypothesis that P2 triggering is responsible for the maintenance of APE1/Ref-1 in the nuclear compartment.

APE1/Ref-1 nuclear translocation upon ATP and H_2O_2 stimulation is Ca^{2+} and PKC-dependent

Previous studies demonstrated that PKC is able to modulate APE1/Ref-1 functions (29) and that a Ca^{2+} ionophore induces APE1/Ref-1 nuclear translocation in rat FRTL-5 thyroid cells (30). It is also known that ATP induces both intracellular Ca^{2+} response and PKC activation (27). We investigated whether inhibition of these two pathways was able to affect APE1/Ref-1 nuclear translocation. When the cells were loaded with the intracellular Ca^{2+} chelator BAPTA-AM (10 μM) 30 min before and during ATP stimulation, the ATP-dependent nuclear translocation of APE1/Ref-1 was totally abolished (Figure 3A, lanes 1–4). Confocal analysis also confirmed that BAPTA-AM inhibited APE1/Ref-1 trafficking to the nucleus (data not shown) demonstrating that intracellular Ca^{2+} mobilization regulated ATP-induced APE1/Ref-1 nuclear translocation. In calcium imaging experiments on fura2-loaded (16) ARO cells, incubation with the intracellular Ca^{2+} chelator BAPTA-AM (10 μM, 30 min) totally prevented

the ATP-induced response, indicating that this non-toxic concentration of the chelator efficiently buffered the Ca^{2+} rise (data not shown). To investigate PKC involvement, we downregulated the enzyme by treating the cells overnight with 1 μM phorbol myristate acetate (PMA) (31). The treatment did not affect the ATP-induced response, in terms of Ca^{2+} mobilization (data not shown), thus ruling out a major involvement of PKC in Ca^{2+} rise. Western blot analysis (Figure 3A, lanes 5–8) clearly demonstrated that, in these conditions, the ATP-dependent APE1/Ref-1 nuclear translocation was totally inhibited. Similar results were obtained with the specific PKC inhibitor bis-indolylmaleimide I, also called GF 109203X (data not shown) (32). By confocal analysis, we observed that in the presence of CHX and PMA, APE1/Ref-1 did not translocate to the nucleus upon ATP stimulation, showing the same amount of nuclear and cytoplasmic localization present at the steady state as well as before each ATP addition (data not shown). Thus, these data clearly demonstrate that intracellular Ca^{2+} mobilization and PKC activation are independently required for APE1/Ref-1 trafficking to the nucleus upon P2 triggering by extracellular ATP.

Previously, we and other groups have observed that ROS exposure induces the neosynthesis and the translocation of APE1/Ref-1 from the cytosol to the nucleus of stimulated cells (1), through a still unknown mechanism. Recently, it has been demonstrated that H_2O_2 production can be triggered by various extracellular stimuli, including G-protein-coupled or -uncoupled receptors activation (33,34), acting as second messenger. With the aim of finding the existence of similarities with the ATP-induced APE1/Ref-1 stimulation, we performed a series of experiments by treating cells with H_2O_2. Time-course of ARO cells exposed to 1 mM H_2O_2 indicated that, similarly to what exerted by exogenous ATP, APE1/Ref-1 activation is associated to two early mechanisms, neosynthesis and its translocation from the cytosol to the nucleus (data not shown). Then, we investigated the role of intracellular Ca^{2+} and PKC in H_2O_2-induced APE1/Ref-1 activation (Figure 3B). ARO cells were pre-treated overnight with PMA,

Figure 2. Purinergic triggering causes APE1/Ref-1 cytoplasm to nucleus translocation and its maintenance in the nuclear compartment in a protein-neosynthesis independent manner. (**A**) ARO cells were cultured in serum-free medium for 48 h and then stimulated or not with ATP for the indicated times and in the presence of the protein synthesis inhibitor CHX (15 μg/ml) 5 min before and during the ATP treatment. After stimulation, nuclear and cytoplasmic extracts were prepared and analysed by western blot analysis for the presence of APE1/Ref-1, as described above. Western blot shown is representative of three. Lower panel, values obtained from densitometric analysis of three independent western blot experiments were reported as histograms. Columns and bars indicate the means ± SD from three independent experiments. Black bars represent the nuclear levels of APE1/Ref-1 and the white dotted bars represent the cytoplasmic APE1/Ref-1 protein levels obtained from densitometric analysis of three independent experiments, normalized versus actin (*, $p \leqslant 0.05$; **, $p < 0.001$ compared with controls). (**B**) ARO cells were treated as in (A). Cells were then fixed and stained for APE1/Ref-1 (green). Nuclei were visualized by propidium iodide counterstaining (red). (**C**). P2 receptor triggering is responsible for APE1/Ref-1 cytoplasm to nucleus translocation induced by ATP treatment. ARO cells were cultured in serum-free medium for 48 h and then stimulated with the indicated amounts of ATP for 10 min. Representative western blot analysis, performed as described in Materials and Methods, of APE1/Ref-1 expression levels of both nuclear and cytoplasmic extracts of ARO cells stimulated or not with decreasing amounts of ATP, as indicated (lanes 2–4). In order to test whether extracellular ATP activates APE1/Ref-1 translocation by activation of P2 cell-surface purinergic receptors, ARO cells were pre-treated with the specific P2 receptor antagonist suramin (100 μM) 30 min before the addition of 100 μM ATP (lanes 5 and 6), or with ATP-γS (lane 7) or with adenosine (lane 8) alone. (**D**). Stimulation of APE1/Ref-1 nuclear translocation by P2Y triggering in ARO cells. ARO cells were cultured in serum-free medium for 48 h and then stimulated with the indicated agonists. Representative western blot analysis, performed as described in Materials and Methods, of APE1/Ref-1 expression levels of nuclear and cytoplasmic extracts of ARO cells is reported. (**E**). P2 receptors expression by ARO cells. Left panels: PCR amplification of cDNAs with $P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$ and GAPDH primer pairs. cDNAs were synthesized from RNA template prepared from ARO cells or MG-63 cells, as a positive control. As indicated, the bands of 259 (lane 1, upper part), 362 (lane 2, upper part), 449 and 530 bp (lanes 2, lower part) correspond to amplification products specific for $P2Y_1$ and $P2Y_2$, $P2Y_4$ and $P2Y_6$ receptors cDNA, respectively. As a control of cDNA quality, GAPDH yields a larger 598 bp band. The control reactions contained not retro-transcribed mRNA instead of template cDNA (lanes 3 and 4). Right panel: western blot analysis of $P2Y_1$ and $P2Y_2$ receptors in ARO cells. Analysis was performed as described in Materials and Methods. To assay for the specificity of the recognized bands, competition experiments with the specific peptides of the two receptors were performed by pre-incubating each antibody for 30 min at room temperature with each specific peptide (lane 3). (**F**). Continuous purinergic triggering controls the subcellular levels of APE1/Ref-1 in ARO cells. ARO cells were treated with the indicated amounts of the ATP/ADPase apyrase and with the P2 receptor inhibitor suramin for the indicated times. Representative western blot analysis, performed as described in Materials and Methods, of APE1/Ref-1 expression levels of nuclear and cytoplasmic extracts of ARO cells is reported.
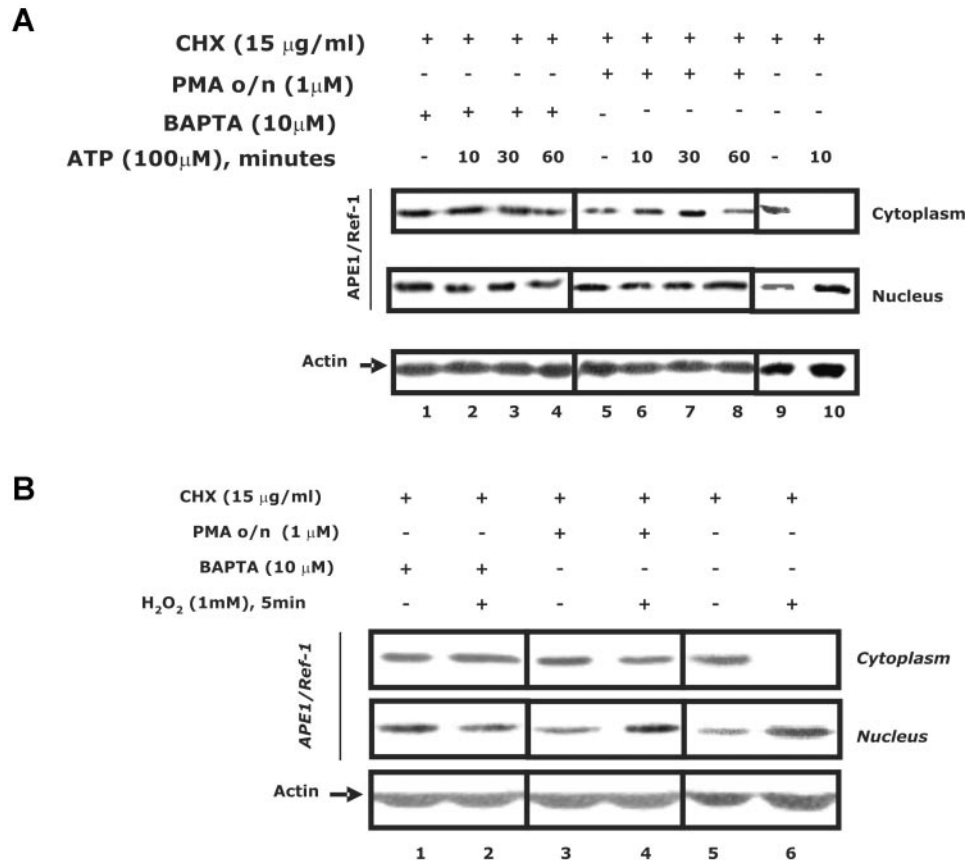
A

B

Figure 3. Intracellular Ca^{2+} mobilization and PKC are involved in APE1/Ref-1 nuclear translocation. (**A**). ARO cells were cultured in serum-free medium for 48 h and then stimulated with ATP for the indicated times. Cells were pre-treated with the intracellular Ca^{2+} chelator BAPTA-AM (10 μM) for 30 min before and during the stimulation time or PKC activation was abolished by incubating the cells with 1 μM PMA o/n and during the stimulation time. Treatments with BAPTA-AM or PMA in the absence of ATP addition were used as controls. After stimulation, nuclear and cytoplasmic extracts were prepared and analysed by western blot for the presence of APE1/Ref-1, as described above. Western blot shown is representative of three. (**B**). H_2O_2-induced APE1/Ref-1 cytoplasm to nucleus translocation is inhibited by intracellular Ca^{2+} mobilization and PKC activation. ARO cells were cultured in serum-free medium for 48 h and then stimulated with H_2O_2 for 5 min. Cells were pre-treated with the intracellular Ca^{2+} chelator BAPTA-AM (10 μM) for 30 min before and during the stimulation time or PKC activation was abolished by incubating the cells with 1 μM PMA o/n and during the stimulation time. Treatments with BAPTA-AM or PMA in the absence of H_2O_2 addition were used as controls. After stimulation, nuclear and cytoplasmic extracts were prepared and analysed by western blot analysis for the presence of APE1/Ref-1, as described above. Western blot shown is representative of three.

to downregulate PKC, and with BAPTA-AM to deplete intracellular Ca^{2+}. After the addition of 1 mM H_2O_2 for 5 min, cytosol and nuclear extracts were prepared and assayed by western blot. We observed that the translocation of APE1/Ref-1 from cytosol to nucleus was evident in cells stimulated with H_2O_2 and in the absence of PMA or BAPTA-AM (Figure 3B, lanes 5 and 6), while in cells treated with these inhibitors the translocation was completely inhibited (Figure 3B, lanes 1–4). These results clearly indicate that, similarly to what seen for the ATP-triggering, H_2O_2-induced translocation of APE1/Ref-1 is also dependent on PKC and calcium ion. This fact suggests that H_2O_2 and ATP trigger the same signal transduction pathway and that a linkage may exist between oxidative stress and involvement of purinergic-triggering in controlling cell functioning.

ATP stimulation promotes ROS generation in ARO cells through NADPH oxidase, which is required for APE1/Ref-1 cytoplasm to nucleus translocation

It has been recently reported that prostate tumour spheroids may generate intracellular ROS, upon purinergic triggering, by
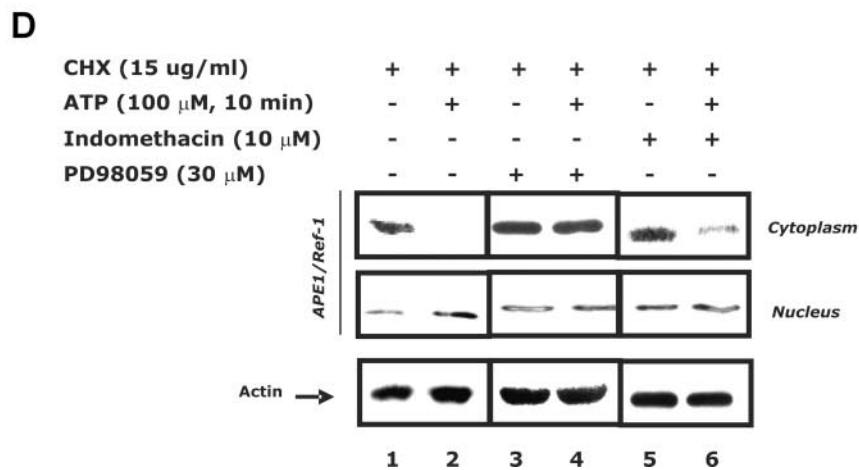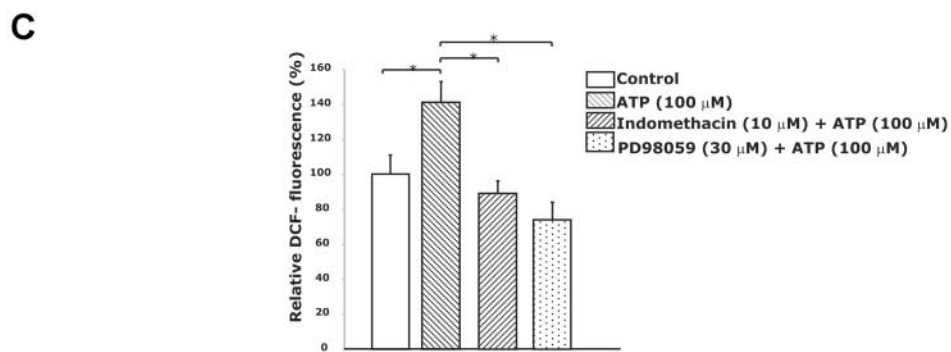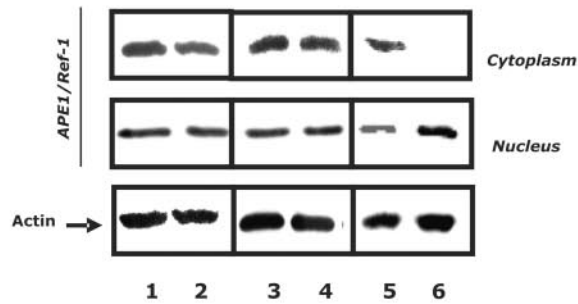
a non-phagocytic NADPH oxidase (18). Therefore, since APE1/Ref-1 is sensitive to intracellular ROS levels, we hypothesized that ATP-induced APE1/Ref-1 translocation in ARO cells could be mediated by intracellular ROS rise. Thus, we tested the ability of ARO cells to produce intracellular ROS upon purinergic triggering. Indeed, incubation of ARO cells with 100 μM ATP for 10 min significantly increased the levels of intracellular ROS (Figure 4A), as evidenced by oxidation of non-fluorescent H_2DCF to the highly fluorescent oxidation product DCF. To test whether the increase of DCF fluorescence reflects an elevation of intracellular ROS levels, we pre-incubated ARO cells with the free radical scavenger *N*-acetyl cysteine (NAC). As it is clear from Figure 4A, 10 mM NAC treatment is able to significantly reduce the relative fluorescence indicating that a generation of intracellular ROS does really occur upon ATP stimulation of the cells.

Several studies indicated a leading role for an NADPH oxidase in generating intracellular ROS in non-phagocytic cells (35). To investigate the possible involvement of an NADPH oxidase in the ATP-mediated ROS generation, ARO cells were pre-incubated with the specific NADPH

A
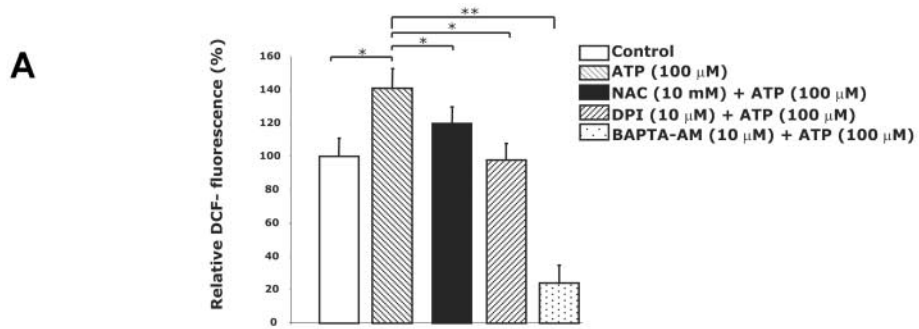
B

CHX (15 μg/ml)		+	+	+	+	+	+
ATP (100 μM, 10 min)		−	+	−	+	−	+
DPI (10 μM)		−	−	+	+	−	−
NAC (10 mM)		+	+	−	−	−	−

C

D

CHX (15 ug/ml)		+	+	+	+	+	+
ATP (100 μM, 10 min)		−	+	−	+	−	+
Indomethacin (10 μM)		−	−	−	−	+	+
PD98059 (30 μM)		−	−	+	+	−	−

oxidase inhibitor diphenylene iodonium chloride (DPI) (36), and intracellular ROS levels were monitored by the use of the ROS indicator H_2DCFDA. DPI treatment (Figure 4A) completely blocked the ATP-induced ROS generation, indicating the involvement of a non-phagocytic NADPH oxidase in ROS generation following purinergic receptor triggering.

Here, we have demonstrated that intracellular Ca^{2+} mobilization upon purinergic triggering by ATP is a fundamental prerequisite for APE1/Ref-1 cytoplasm to nucleus translocation. Thus, we tested the involvement of $[Ca^{2+}]_i$ rise in ROS generation. ATP elicits a transient $[Ca^{2+}]_i$ increase in ARO cells (data not shown). When ARO cells were loaded with the intracellular Ca^{2+} chelator BAPTA-AM, ROS generation following ATP was completely blunted and the ROS levels dropped to a value far under the basal level, suggesting that Ca^{2+} mobilization is also required for the basal production of intracellular ROS (Figure 4A). The intracellular Ca^{2+} mobilization, as followed by calcium imaging experiments on fura2-loaded ARO cells, was not abolished in the presence of NAC, suggesting that it does not depend on the generation of intracellular ROS (data not shown). Thus, we can conclude that Ca^{2+} mobilization seems to be the most important regulatory mechanism able to control the intracellular ROS production of ARO cells. We, therefore, tested the role of NADPH oxidase with respect to ATP-mediated APE1/Ref-1 cytoplasm to nucleus translocation (Figure 4B). We performed experiments of ATP stimulation in cells pre-treated with CHX (15 μg/ml) in order to follow only the translocation event of APE1/Ref-1. Indeed, APE1/Ref-1 translocation is completely prevented by treatment of the cells with the NADPH oxidase inhibitor DPI and the ROS scavenger NAC. Therefore, ATP-induced APE1/Ref-1 translocation requires active production of intracellular ROS by an NADPH oxidase.

The non-phagocytic NADPH oxidase has been previously demonstrated to be activated by Ca^{2+}-activated phospholipase A_2 (PLA_2) (18). In order to investigate whether this is also the case in our cell system and whether it is related to APE1/Ref-1 translocation, ARO cells were pre-incubated with PLA_2 inhibitor indomethacin, and ROS generation and APE1/Ref-1 translocation were analysed (Figure 4C and D, respectively). Indeed PLA_2 inhibition was responsible for blockage of ROS production together with a partial, though significant, inhibition of APE1/Ref-1 cytoplasm to nucleus translocation (Figure 4D, lanes 5 and 6).

It has been previously demonstrated, in several cell systems, that extracellular regulated kinases (ERKs) contribute to functionally activate the NADP(H) oxidase by phosphorylating different subunits (37,38). In order to check whether

ERK-1/2 may also be involved in ATP-induced activation of ROS production and APE1/Ref-1 translocation, both phenomena were analysed. Data are reported in Figure 4C and D. PD98059 (a specific inhibitor of ERK-1/2 phosphorylation) pre-treatment was able to both block intracellular ROS production (Figure 4C) and APE1/Ref-1 cytoplasm to nucleus translocation (Figure 4D, lanes 3 and 4). Therefore, ERKs signalling is also responsible for the activation of intracellular ROS production by ATP stimulation.

Inhibition of intracellular Ca^{2+} mobilization and ROS production block APE1/Ref-1 nuclear translocation and its functional activation

In order to test whether the stimulatory effect of ATP treatment has consequences on APE1/Ref-1 functions, we first tested its DNA repair activity. APE1/Ref-1 enzymatic activities, on nuclear extracts from ARO cells stimulated or not with extracellular ATP, were assayed, as described previously (6). The assay uses a radiolabelled oligonucleotide (26mer) containing an artificial tetrahydrofuran AP site which, when cleaved by AP endonuclease, produces a labelled 14mer. The same amounts of nuclear extracts from non-stimulated and ATP-stimulated ARO cells were assayed for endonuclease activity and are shown in Figure 5A. The increased nuclear accumulation of APE1/Ref-1 upon 10 min of ATP stimulation was paralleled by an increasing AP endonuclease activity, as measured with the oligonucleotide cleavage assay (Figure 5A, compare lane 4 with lane 3). In order to test the role of intracellular Ca^{2+} mobilization and ROS generation on APE1/Ref-1 DNA repair functions, we measured the effects of BAPTA-AM and DPI on APE1/Ref-1 enzymatic activities, by using the same assay. As it is evident from Figure 5A, when cells were pre-treated with BAPTA-AM (lane 5) or DPI (lane 6), the increase in APE1/Ref-1 cleavage activity upon ATP stimulation was completely prevented.

In order to test whether APE1/Ref-1 activation following ATP stimulation has a functional relevance as a redox modulator, the activity of the well-established redox-regulated transcription factor AP-1 was assessed. ARO cells were treated with CHX as described above and further stimulated for 10 min with 100 μM ATP. Then, nuclear extracts were prepared and assayed for AP-1 DNA-binding activity by EMSA analysis (Figure 5B). Early time stimulation by ATP is able to significantly enhance the DNA-binding activity of AP-1, demonstrating a 3-fold increase in specific DNA-binding activity (compare lane 2, unstimulated, and lane 3, stimulated). To test the causal role of APE1/Ref-1 in the ATP-induced

Figure 4. ATP-induced APE1/Ref-1 nuclear translocation is dependent on ROS produced by ARO cells. (**A**). Elevation of intracellular ROS following treatment of ARO cells with ATP is inhibited by free radical scavenger and depends on the activation of non-phagocytic NADPH oxidase in a Ca^{2+}-dependent manner. ARO cells were cultured in serum-free medium for 48 h and then stimulated with ATP for 10 min. Oxidation of non-fluorescent H_2DCF to fluorescent DCF was followed as indirect measure of intracellular ROS levels. After incubation of ARO cells for 10 min with ATP, the amount of fluorescent DCF was monitored. In inhibitory experiments, ARO cells were pre-incubated with either 10 mM NAC (2 h), 10 μM DPI (10 min) or 10 μM BAPTA-AM (30 min) before ATP treatment. Subsequently, the DCF fluorescence was monitored, as above (Statistical analysis by ANOVA *, $p \leqslant 0.05$; **, $p < 0.001$). (**B**). Rise in intracellular ROS levels is responsible for ATP-induced APE1/Ref-1 nuclear translocation. ARO cells were treated as in (A). After stimulation, nuclear and cytoplasmic extracts were prepared and analysed by western blot analysis for the presence of APE1/Ref-1 and actin, as described above. Western blot shown is representative of three. (**C**). Activation of intracellular ROS production upon ATP stimulation by NADPH oxidase is dependent on ERKs phosphorylation and PLA_2. ARO cells were cultured in serum-free medium for 48 h and then stimulated with ATP for 10 min. In inhibitory experiments, ARO cells were pre-incubated for 1 h with either 10 μM indomethacin as PLA_2 inhibitor or 30 μM PD98059, as ERK-1/2 phosphorylation inhibitor; the cells were then incubated for 10 min with 100 μM ATP. ROS levels were monitored as described above (Statistical analysis by ANOVA *, $p \leqslant 0.05$; **, $p < 0.001$). (**D**). APE1/Ref-1 nuclear translocation is dependent on activation of ERK-1/2 and PLA_2. ARO cells were treated as in (C). After stimulation, nuclear and cytoplasmic extracts were prepared and analysed by western blot analysis for the presence of APE1/Ref-1 and actin, as described above. Western blot shown is representative of three.
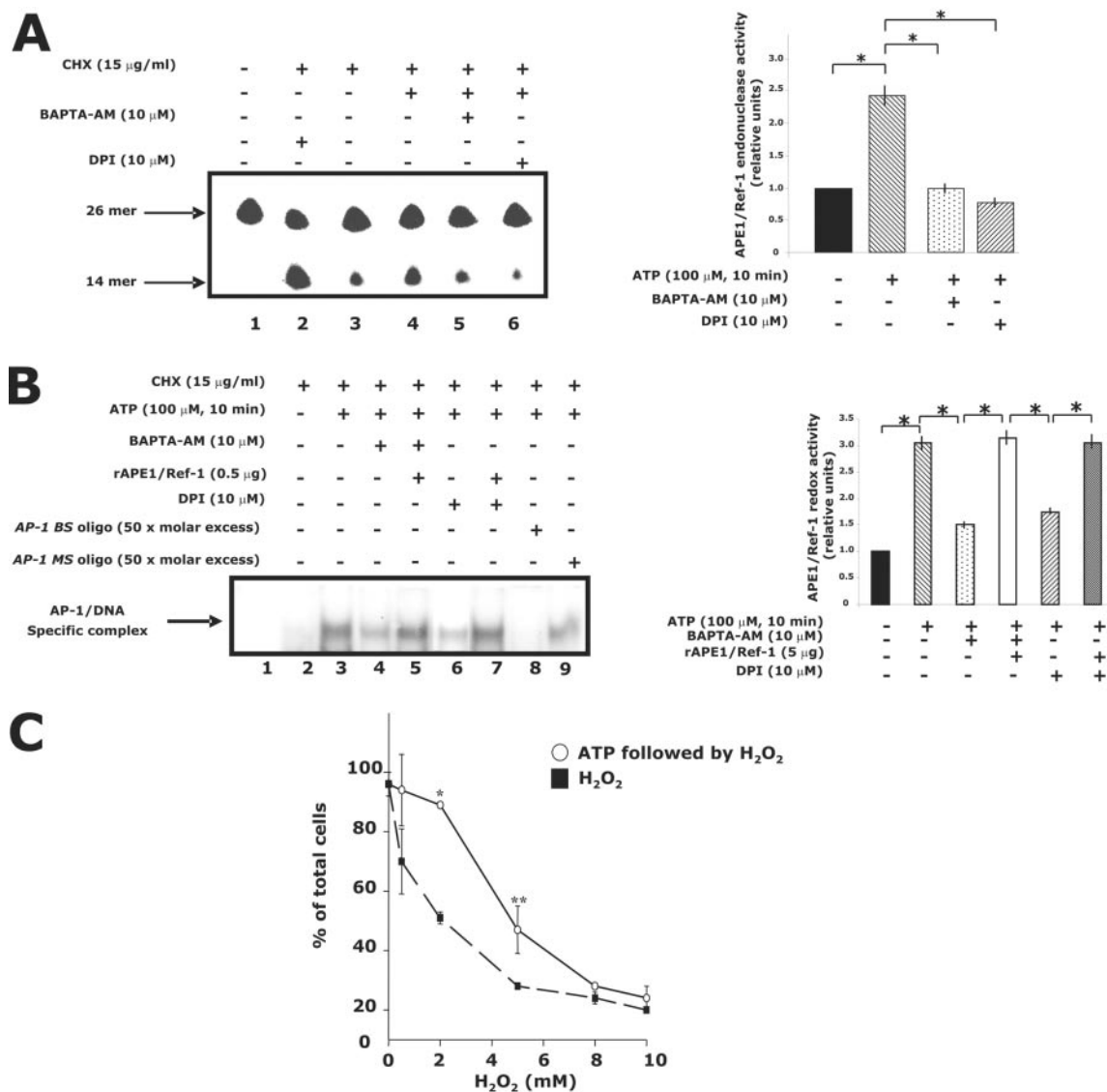
Figure 5. Inhibition of intracellular Ca^{2+} mobilization and ROS production blocks APE1/Ref-1 nuclear translocation and its functional activation. (**A**). Left: blockage of intracellular Ca^{2+} mobilization and ROS production prevents early activation of base excision repair activity of APE1/Ref-1 upon ATP stimulation. ARO cells were cultured in serum-free medium for 48 h and then stimulated with ATP for 10 min. To blunt the effects of Ca^{2+} signals, cells were treated with the intracellular Ca^{2+} chelator BAPTA-AM and to block the intracellular ROS production, cells were treated with DPI. Thereafter, cells were treated for 10 min with 100 μM ATP. Lane 1, oligonucleotide alone. Lane 2, endonuclease activity of recombinant APE1/Ref-1 (5 ng) as a positive control. Lane 3, endonuclease activity of 50 ng of unstimulated ARO cells. Lane 4, endonuclease activity of 50 ng of stimulated ARO cells. Lane 5, endonuclease activity of 50 ng of stimulated ARO cells after pre-treatment with BAPTA-AM (10 μM) for 30 min. Lane 6, endonuclease activity of 50 ng of stimulated ARO cells after pre-treatment with DPI (10 μM) for 10 min. For simplicity, only a dose, from a scalar dilution experiment, of nuclear extract is reported. Right: values representing the relative endonuclease activity of endogenous APE1/Ref-1 were reported as histograms and reflect the percentage of cleavage of the radiolabelled AP site-containing oligonucleotide. Columns and bars indicate the means ± S.D. of three different experiments (*, $p \leqslant 0.05$ compared with controls by Student's *t*-test). (**B**). Left: blockage of intracellular Ca^{2+} mobilization and ROS production prevents early activation of AP-1 binding activity upon ATP stimulation dependent on APE1/Ref-1. To blunt the effects of Ca^{2+} signals and intracellular ROS generation, cells were treated with BAPTA-AM (lanes 4 and 5) and with DPI (lanes 6 and 7) as described in A, respectively. Thereafter, cells were treated for 10 min with 100 μM ATP. Then, cells were collected and lysed to obtain nuclear extracts that were subsequently used for EMSA analysis with the ^{32}P-labelled specific oligo called *AP-1 BS*. An aliquot of 5 μg of each nuclear extract was incubated with 200 fmol of labelled probe in the presence of 500 ng of calf thymus DNA as aspecific competitor for 30 min at room temperature and then analysed by native 5% PAGE. After drying, the gel was exposed o/n at −80°C for autoradiography. To test for the specific protein/DNA complex, a competition assay was performed, on ATP-stimulated nuclear extracts, with a molar excess of the cold specific *AP-1 BS* (lane 8) and with a mutant binding site called *AP-1 MS* (lane 9). In order to test for the real involvement of APE1/Ref-1 in the blockage of the ATP-induced AP-1 DNA-binding activity exerted by both BAPTA-AM and DPI, nuclear extracts from the ATP-stimulated cells treated with the inhibitors were re-added of 0.5 μg of recombinant purified APE1/Ref-1 protein (rAPE1/Ref-1) (lanes 5 and 7). Lane 1 represents the oligonucleotide alone; lane 2 represents the AP-1 DNA-binding activity in basal conditions; lane 3 represents the AP-1 DNA-binding activity of ATP-treated ARO cells. Data are representative of three independent experiments. Right: values representing the relative DNA-binding activity of AP-1 were reported as histograms. Columns and bars indicate the means ± SD of three different experiments (*, $p \leqslant 0.05$ compared with controls by Student's *t*-test). (**C**). Protection by ATP of H_2O_2-evoked cell death in ARO cells. ARO cells were pre-incubated with ATP (100 μM) for 30 min and then exposed to various concentrations of H_2O_2 for 1 h. Protein neosynthesis was blocked by pre-treatment of the cells for 5 min with CHX (15 μg/ml) and for the whole duration of the experiment. The cell viability was evaluated by the MTT assay. The plots show mean ± S.D. of triplicate measurements. Values were normalized to total cell number (basal) and the cell viability was expressed as percentage of total cell. Asterisks show significant differences from the control response (*$p = 0.011$, **$p = 0.0221$, Student's *t*-test).

AP-1 DNA-binding activity induction, we performed experiments by using an APE1/Ref-1 immunodepletion/reconstitution assay (data not shown) (39). These data suggest that, indeed, APE1/Ref-1 nuclear translocation acts as a physiological activator of AP-1 DNA-binding activity in response to ATP treatment of ARO cells, thus providing a molecular explanation for the early activation of AP-1 DNA-binding activity demonstrated in several cell types upon purinergic triggering (14,15).

Then, we tested whether activator pathways leading to APE1/Ref-1 translocation (i.e. Ca^{2+} mobilization and intracellular ROS production) by ATP have a functional relevance, in terms of co-activator role on AP-1. ARO cells were treated in the presence of CHX as described above, stimulated for 10 min with 100 µM ATP in the presence of inhibitors of APE1/Ref-1 translocation (DPI and BAPTA-AM). Nuclear extracts were prepared and assayed for AP-1 DNA-binding activity by EMSA analysis (Figure 5B, lanes 4–7). Indeed, blocking the intracellular calcium mobilization by BAPTA-AM pre-treatment substantially prevents the ATP-induced activation of AP-1 DNA-binding activity (compare lane 4 with lane 3). The exogenous application of 0.5 µg of recombinant purified APE1/Ref-1 (rAPE1/Ref-1) to the nuclear extract from BAPTA-AM-treated cells efficiently restored the ATP-induced AP-1 DNA-binding activity (lane 5), demonstrating that the blockage due to the intracellular calcium chelator is exerted by means of APE1/Ref-1. Similar results were obtained when cells were pre-treated with the NADPH-oxidase inhibitor DPI (lanes 6 and 7). These results clearly demonstrate that either inhibition of intracellular ROS production or Ca^{2+} mobilization is able to prevent AP-1 stimulation induced by ATP by means of APE1/Ref-1 nuclear translocation.

Therefore, we can conclude that the two well-known APE1/Ref-1 functions (as a DNA repair enzyme and as a redox co-activator) are exerted by ATP stimulation through APE1/Ref-1 nuclear translocation.

Protection by ATP of H_2O_2-evoked cell death in ARO cells

It is commonly accepted that APE1/Ref-1 functional activation is required for protecting cells from oxidative injuries (1,40). Moreover, it has been recently demonstrated that over-expression of APE1/Ref-1 is able to prevent the H_2O_2-induced cell death in different cells systems (41,42). Therefore, we tested whether ATP stimulation was able to protect cells by H_2O_2-evoked cell death. ARO cells were stimulated or not for 30 min with 100 µM ATP, in the presence of CHX (15 µg/ml), and treated for an additional 1 h with increasing amounts of H_2O_2, and cell viability was then assayed by MTT analysis (Figure 5C). H_2O_2 treatment caused a concentration-dependent decrease in cell viability of ARO cells, due to cell death. When incubated for 1 h with 5 mM H_2O_2, the cell viability was almost halved and then gradually decreased to ~20% of the non-treated control level. Figure 5C shows that pre-treatment with ATP significantly reduced the H_2O_2-induced cell death exerted at 2 and 5 mM dose of H_2O_2. Therefore, these data reinforce the functional relevance of our findings on APE1/Ref-1, suggesting that activation of APE1/Ref-1 by extracellular ATP is associated to a protection towards H_2O_2-induced cell death exerted by purinergic

triggering. Further experiments are in progress to demonstrate the causal involvement of APE1/Ref-1 activation in ATP-induced protection to H_2O_2 treatment.

DISCUSSION

The present study was undertaken to investigate whether the activation of P2 receptors by extracellular nucleotides is involved in the regulation of signal transduction mechanisms leading to APE1/Ref-1 activation. The results demonstrate that extracellular ATP stimulates a Ca^{2+}-dependent ROS production pathway ultimately leading to (i) activation of APE1/Ref-1 cytoplasm to nucleus translocation and maintenance in the nuclear compartment in a protein-neosynthesis independent manner (occurring at very early times upon stimulation, i.e. within 10 min) and (ii) stimulation of its expression at later times (30 and 60 min). The early increase in nuclear accumulation is well paralleled by a functional activation of APE1/Ref-1 as a DNA repair enzyme over abasic sites, and as a redox co-activator of AP-1 DNA-binding activity. These results suggest a likely explanation of the molecular mechanisms at the basis of the protective role exerted by ATP stimulation of ARO cells against H_2O_2-induced cell death. Obviously, the causal role of APE1/Ref-1 nuclear accumulation upon purinergic triggering in controlling these biological effects should be demonstrated. However, at present, this is impossible since it would require a dominant-negative form of the protein able to inactivate the nuclear translocation of the endogenous APE1/Ref-1 protein. Unfortunately, at present, the molecular mechanisms responsible for regulating APE1/Ref-1 cytoplasm to nucleus translocation are poorly understood (i.e. post-translational modifications, interaction with shuttling proteins, etc.). Conversely, the importance of APE1/Ref-1 intracellular levels in controlling cell proliferation and DNA repair has been already well documented by different works using gene silencing techniques (41–43). A very recent paper shed some lights showing that APE1/Ref-1 nuclear translocation is an active process involving Karyopherin 2α (44). These authors also showed the existence of nuclear export process responsible for regulating the APE1/Ref-1 nuclear level. In light of our data, showing that P2-induced signalling plays an important role in controlling nuclear maintenance of the protein, we can hypothesize that this process could be an inhibitory target of P2 pathway here delineated.

We have characterized the mechanisms responsible for the activatory effects on APE1/Ref-1 and found that purinergic signalling is mainly exerted by intracellular ROS production by non-phagocytic NADPH oxidase in a calcium-dependent manner; however, additional MAPK- and/or PKC-dependent mechanisms could also be involved (see Figure 6). The involvement of NADPH oxidase in the activation of APE1/Ref-1 suggests the existence of an autoregulatory loop between these two proteins. In fact, the non-phagocytic NADPH oxidase is an enzyme composed of multiple membrane-associated (Mox and p22^{phox}) and cytosolic components (p67^{phox}, p47^{phox}, Rac1) and catalyses the transformation of the molecular oxygen to the superoxide anion by transferring an electron from the substrates NADH or NADPH (45). Moreover, in thyroid cells, it represents the
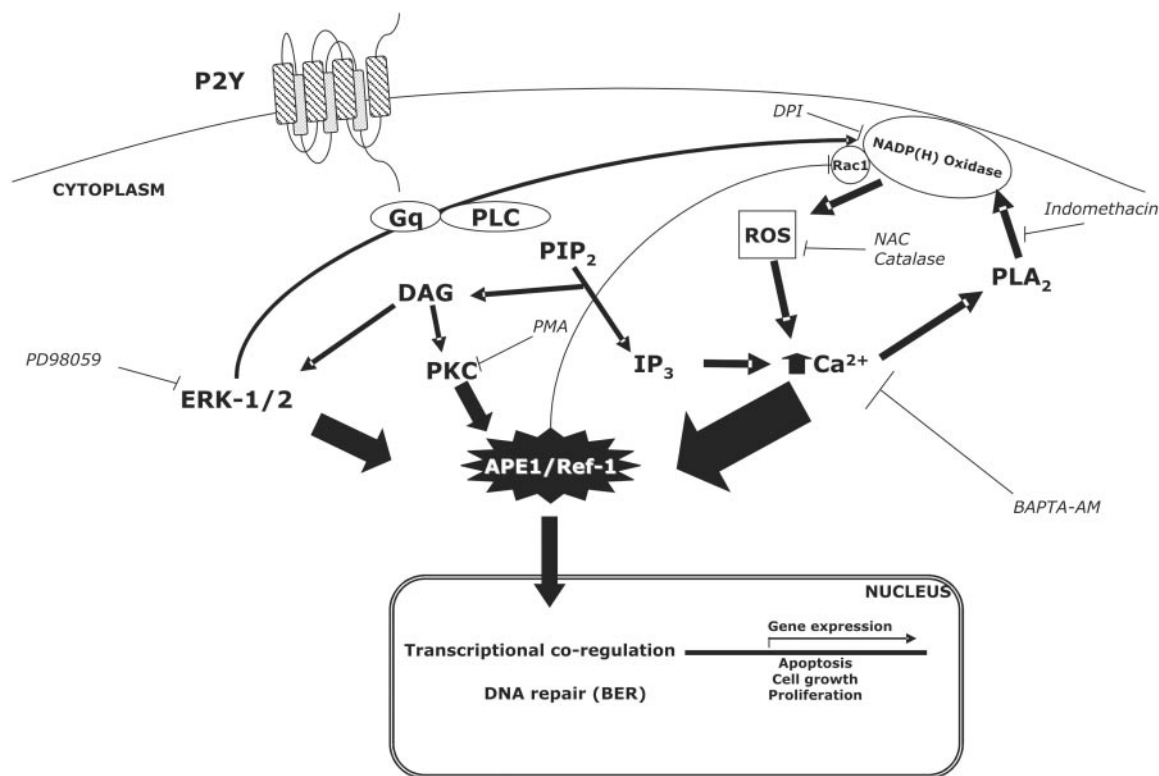
Figure 6. Model of the activatory role of purinergic triggering on APE1/Ref-1 subcellular localization and functions.

major source of H_2O_2 necessary for thyroid hormone biosynthesis (46). Functional activation of this enzyme is controlled by Rac1, a small GTPase exerting regulatory and assembling functions on the oloenzyme itself (47). It has been recently demonstrated that APE1/Ref-1 may inhibit Rac1 activity *in vivo*, in different cell models, thus controlling intracellular ROS production (48,49). Therefore, we can hypothesize that APE1/Ref-1 may also be involved in extinguishing the NADPH-mediated ROS production induced by P2Y triggering (see Figure 6). Work is in progress to address this issue. Since APE1/Ref-1 and NADPH oxidase are ubiquitously expressed, and purinergic triggering is particularly important for endothelial, fibroblastic and smooth muscle cells in the vascular system (50), the present findings may be therapeutically relevant.

At present, receptors for ATP and other nucleotides have been found in almost all cell types and have wide physiological involvement both in normal and in pathological conditions. Though the effects of extracellular nucleotides in endocrine cells are largely unexplored and even if the thyroid gland is mainly regulated by thyrotropin, it is also under regulation by sympathetic and parasympathetic nerves, whose terminals also reach thyroid follicles (51). Therefore, in principle, ATP released from nerve terminals may also modulate thyroid functions. In particular, investigations in thyroid cell cultures have shown that ATP may act as true agonist being involved in some physiological responses, such as regulation of iodide fluxes and generation of H_2O_2 (52) and may act as co-mitogen in normal rat FRTL-5 thyroid cell lines (15) through up-regulating the expression of proto-oncogenes,

such as c-Fos and the enhancement of DNA-synthesis (53). In thyroid cells, the molecular mechanism of such activations described a central role for Ca^{2+} fluxes evoked by ATP and activation of PLA_2 as consequences of the ATP-induced stimulation of phospholipase C. However, the effects of the interactions of these two pathways, in terms of molecular targets, have not yet been thoroughly investigated in thyroid cells. Interestingly, very recent data demonstrated that transformed thyroid cell lines show disturbances in purinergic Ca^{2+} signalling (54). Whether alteration in purinergic signalling plays a role in tumourigenic process is unknown, at present. Our data, obtained under continuous treatment with apyrase and suramin, showed that purinergic triggering is responsible for the maintenance of high cytoplasmic levels of APE1/Ref-1 protein in the anaplastic tumoural thyroid ARO cell line. These data, together with the observation that ATP stimulation exerts protective role on H_2O_2-induced cell death, provide two important suggestions: (i) alterations of the intracellular amounts of APE1/Ref-1 found in several tumours, including thyroid tumours (1), may be due to an imbalance in purinergic receptor signalling system and (ii) purinergic receptors alterations may be an early event occurring during the tumourigenic process and may provide the tumour cell with higher protective mechanisms towards oxidative injuries. In this light, much more attention should be paid in order to better understand the role, if any, of purinergic receptor signalling in controlling specific differentiative steps, as recently demonstrated by the finding that a coordinated expression of several purinergic receptors of different classes occur during skeletal muscle development (55).

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