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Extracellular ATP-induced mitogenic response in rat kidney is mediated via purinergic P2X7Rs

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Abstract: Extracellular ATP is a potent signaling molecule that regulates different biological activities including proliferation, differentiation as well as cell death. Several studies have implicated extracellular ATP as a mitogenic agent in various cells *in vitro*. Herein, we aimed to investigate the involvement of ATP in mitogenic processes *in vivo* via stimulation of P2X7Rs in rat kidney. Results from the current study showed that administration of ATP caused rapid activation of ADAM-17 with subsequent phosphorylation of EGFR and ERK-1/2. Cyclin B1 expression in rat kidney was also increased following ATP administration. Interestingly, a marked decrease in renal ADAM-17 levels as well as EGFR and ERK-1/2 phosphorylation was noticed when the animals were pretreated with the purinergic P2X7R antagonist, A 438079 before ATP administration. Moreover, ATP administration significantly increased serum levels of NO, an effect that was significantly ameliorated by A 438079 pretreatment. Collectively, current findings demonstrate the ability of ATP to activate the ADAM-17-mediated EGFR/ERK-1/2 phosphorylation and to enhance the expression of the cell proliferation gene cyclin B1, an effect that is largely mediated via P2X7Rs and involves NO modulation. Thus, the safety of ATP supplementation as an ergogenic aid for muscle building and performance enhancement should be reassessed.

Keywords: ATP; P2X7Rs; A 438079; proliferation; kidney.

1. INTRODUCTION

In addition to powering cellular processes, intracellular adenosine 5′ triphosphate (ATP) can act as a key messenger molecule for cell-to-cell communication once released into the extracellular space [1]. The broad extracellular functions of ATP are chiefly mediated via membrane purinergic receptors (P2Y and P2X) pervasively present in various cell types [2]. The purinergic P2X receptor is an ion channel receptor while the P2Y receptor is coupled to G protein [2]. ATP signaling between cell types in the body regulates proliferation, differentiation as well as cell death [2-5].

A disintegrin metalloenzyme-17 (ADAM-17), initially identified as TACE "the tumor necrosis factor-alpha converting enzyme", is the major sheddase of epidermal growth factor (EGF)-like growth factors that activate EGF receptor-dependent proliferative signaling pathways like the extracellular signal regulated kinase-1/2 (ERK-1/2) mitogen activated protein kinase (MAPK) pathway [6,7]. ERK-1/2 acts as a crucial regulator of cellular proliferation via its effects on protein synthesis and cell cycle entry. In the G2/M transition, the nuclear translocation of cyclin B1 is regulated by ERK-1/2-mediated phosphorylation of the cytoplasmic retention sequence of cyclin B1 [8]. In addition, ERK-1/2 phosphorylates and activates p90(rsk) which in turn down-regulates myelin transcription factor 1, leading to the activation of p34(cdc2)/cyclin B complex [9].

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While ATP's mitogenic role has been well documented *in vitro*, the *in vivo* role in animal models has not been yet clarified. Thus, in this study, we test the possible modulatory effect of ATP on the mitogenic EGFR/ERK-1/2 signaling cascade emphasizing the role of P2X7Rs on the ATP-mediated mitogenic response in rat kidney.

2. METHODS

2.1. Animals

Male Sprague-Dawley rats (body weight 180-200 g) were acquired from Nile Co., Cairo, Egypt. Rats were accommodated in the animal house of Faculty of Pharmacy (Girls), Al-Azhar University (12-hour dark/light cycle with precise control of humidity and temperature). Water and a standard diet were delivered *ad libitum*. The animals were raised for 7 days before the treatments for acclimatization. This experiment was done according to the Ethics Committee of Faculty of Pharmacy (Girls), Al-Azhar University (Approval number: 177/2017) in compliance with the standards of Principles of Laboratory Animal Care (NIH Publications No. 85-23, revised 2011).

2.2. Chemicals, antibodies and kits

PEAK ATP® was purchased from TSI USA, Inc. (Missoula, MT) in the form of capsules containing 400 mg of ATP as the disodium salt. The encapsulated powder was dispersed in saline (10 mg/mL). The P2X7R antagonist, A 438079 was obtained from Sigma-Aldrich Co. (St. Louis, MT, USA) and was first dissolved in dimethyl sulfoxide (DMSO) at 20X stock solution then diluted in saline to obtain a solution with a concentration equal 1 mg/mL. Phosphorylated ERK-1/2 (p-ERK-1/2), total ERK-1/2 (t-ERK-1/2), phosphorylated EGFR (p-EGFR), total EGFR (t-EGFR), cyclin B1 and β-actin antibodies were procured from Thermo Fisher Scientific Inc. (Waltham, MA, USA). Anti-gaot HRP-linked IgGs were acquired from Sigma-Aldrich Co., St. Louis, MT, USA. TRIzol reagent and SuperScript II reverse transcriptase kits were purchased from Invitrogen, Thermo Fisher Scientific Inc. (Waltham, MA, USA). ADAM-17 ELISA kit was obtained from Lifespan biosciences Inc., WA, USA. A nitric oxide (NO) assay kit was obtained from Biodiagnostics, Egypt.

2.3. Experimental design

2.3.1. Design No. 1

To test the effect of ATP on ERK-1/2 signaling activation, the animals (6 rats per group) received a single intraperitoneal dose of ATP, 3.3 mg/kg [10] for different periods (1, 2, 4, 8, 10, and 24 hours). Control animals were administered saline, the vehicle of ATP. At the specified periods, rats were euthanized through cervical dislocation. Immediately after death, the kidney was excised, rinsed with ice-cold PBS, and stored at -80ºC for determination of p-ERK-1/2.

2.3.2. Design No. 2

To explore the implication of P2X7Rs in ATP-induced ERK-1/2 signaling cascade activation, animals were separated randomly into 5 groups (6 rats per group) and treated intraperitoneally with a single dose of either saline **(Control group)**, DMSO; the vehicle of the P2X7R antagonist, A 438079 **(DMSO group)**, ATP; 3.3 mg/kg **(ATP group)**, A 438079; 3 mg/kg **(A 438079 group)** [11] or A 438079 one hour before ATP administration **(ATP + A 438079 group)**. Four hours later (based upon the results of study No. 1), the rats were sacrificed, and the kidney was excised, rinsed with ice-cold PBS, and stored at -80ºC for determination of ADAM-17, p-EGFR, t-EGFR, p-ERK-1/2 and t-ERK-1/2.

2.3.3. Design No. 3

Here we examined whether ERK-1/2 phosphorylation induced by ATP could be translated into an increment of the G2/M regulator, cyclin B1 expression. If so, what is the role of P2X7Rs in the ATP-induced ERK-1/2 mitogenic signaling cascade? In addition, the potential modulatory effect of ATP on the serum level of NO was also examined. The animals were administered either saline, DMSO, ATP, A 438079, or A 438079 in combination with ATP. Twenty-four hours later, blood samples were collected through the retro-orbital plexus. The serum was then separated and used for biochemical evaluation of NO. Then, animals were sacrificed, and the kidney was excised, rinsed with ice-cold PBS, and stored at -80ºC for later analysis of the relative mRNA and associated protein expression of cyclin B1.

2.4. Western blotting

Western blotting analysis was utilized to detect p-ERK-1/2, t-ERK-1/2, p-EGFR, t-EGFR, cyclin B1, and β-actin as described previously [12]. Briefly, ice-cold RIBA lysis buffer was used for total protein extraction from kidney homogenates (1:10 w/v homogenate was prepared using ice-cold 0.1M phosphate buffer, pH 7.4 and centrifuged (3000 g) for 20 min. at 4 °C) and quantified using Bradford

Protein Assay Kit. Protein extracts were denatured using Laemmli sample buffer and separated using 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and blotted on polyvinylidene fluoride (PVDF) membranes (Millipore, USA). Then, membranes were blocked in TBS-Tween buffer containing 3% BSA and probed overnight at 4 °C with the relevant primary antibody at (1:200-1:1000) dilution. Equal loading of protein was demonstrated by probing the membranes with β-actin monoclonal antibody (1:10000). After washing in TBS-Tween buffer, the membranes were incubated again with horseradish peroxidase-conjugated anti-goat antibody (1:10000) for 30 minutes. Finally, blots were visualized by enhanced chemiluminescence (ECL kit; Amersham Pharmacia, Little Chalfont, UK) and quantified by scanned densitometry and ImageJ analysis system.

2.5. Quantitative real-time (qRT)-polymerase chain reaction

Cyclin B1 mRNA expression was determined as described formerly [13]. Briefly, total RNA was extracted using TRIzol reagent (50 mg of kidney tissue was homogenized in 1 mL of TRIzol reagent and then incubated for 5 min. - 0.2 mL of chloroform was added for 2–3 min and the mixture was centrifuged (12,000 g) for 15 min. at 4°C to separate a colorless upper aqueous phase containing the extracted RNA). Then, total RNA was reverse transcribed to the cDNA with a SuperScript II reverse transcriptase according to the manufacturer's recommendations. Amplification and detection were done using the PRISM 7700 Sequence Detector (PE Applied Biosystems, Foster City, CA). The primers sequence from 5′-3′ used in this analysis were as follows: Cyclin B1 forward, AAAGGCGTAACTCGAATGGA; Cyclin B1 reverse, CCGACCTTTTATTGAAGAGCA; GAPDH forward, TCCCTCAAGATTGTCAGCAA; GAPDH reverse, AGATCCACAACGGATACATT. The 5′ reporter dye for the cyclin B1 probes is 6-carboxy fluorescein and for GAPDH probes is 2′,7′-dimethoxy-4′,5′-dichloro-6-carboxyfluorescein . The 3′ quencher dye for both probes is N, N, N′, N′-tetramethyl-6-carboxyrhodamine. Relative changes in cyclin B1 gene expression levels were quantified as the ratio to GAPDH.

2.6. Assessment of ADAM-17 level

The renal level of ADAM-17 was measured using an ELISA kit (raised against rat ADAM-17) following the manufacturer's instructions.

2.7. Assessment of serum nitric oxide level

The serum level of NO was measured using the commercial colorimetric kit (Cat No. NO 2532) purchased from Biodiagnostic Co. (Cairo, Egypt) following the manufacturer's guidelines.

2.8. Statistical Analysis

All the results are expressed as the mean values ± SEM and analyzed using GraphPad Prism (ISI®, USA) software (version 5), applying either unpaired student's t-test or a one-way ANOVA then Tukey as a post-hoc test. Significance was accepted at a P value less than 0.05.

3. RESULTS

3.1. Effect of extracellular adenosine triphosphate (e-ATP) on ERK-1/2 signaling cascade

First, we explored the potential effect of ATP on the ERK-1/2 signaling cascade. Western blotting analysis revealed that ATP administration augments ERK-1/2 phosphorylation in kidney tissue in a time-dependent manner with the peak ERK-1/2 phosphorylation recognized after 4h of ATP administration (Figure 1).

3.2. Effect of extracellular adenosine triphosphate (e-ATP) or/and the P2X7R antagonist (A 438079) on a disintegrin and metalloproteinase-17 (ADAM-17) activity in rat kidney

As shown in Figure (2), ATP-induced activation of ADAM-17 was highly diminished in the presence of A 438079 indicating that P2X7Rs are implicated in ADAM-17 activation induced by ATP.

3.3. Effect of extracellular adenosine triphosphate (e-ATP) or/and the P2X7R antagonist (A 438079) on epidermal growth factor receptor (EGFR) phosphorylation in rat kidney

As indicated in Figure (3), ATP administration significantly enhanced the phosphorylation of EGFR. This effect was considerably diminished by pretreatment of animals with the P2X7R antagonist, A 438079 specifying the role of P2X7Rs in ATP-triggered EGFR phosphorylation in rat kidney.

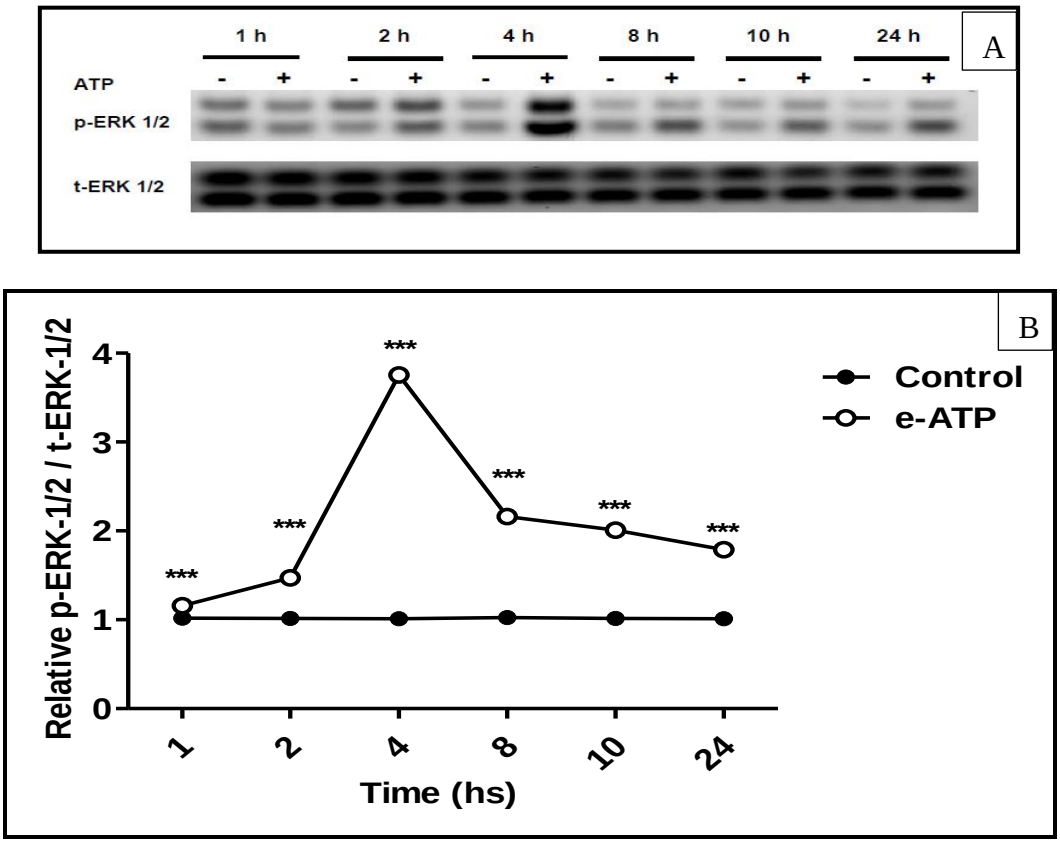

Figure 1. Extracellular ATP triggers ERK-1/2 phosphorylation in a time-dependent manner.

- Total kidney extracts from rats treated intraperitoneally with either saline (-) or e-ATP (+) for the specified periods were subjected to Western blotting analysis (A) and probed with anti-p-ERK-1/2. For ascertainment that the total level of ERK-1/2 remained unchanged, blots were stripped and reprobed with anti-ERK-1/2. The lower panel (B) shows data from densitometric analysis of p-ERK-1/2 relative to t-ERK-1/2 level. - Values are represented as mean ± SEM. (n=6).
- *** indicates a significant difference from the control group at the same time at $P < 0.001$ using the unpaired student's t-test.
- e-ATP, extracellular adenosine triphosphate; p-ERK-1/2, phosphorylated extracellular signal regulated kinase-1/2; t-ERK-1/2, total extracellular signal regulated kinase-1/2.

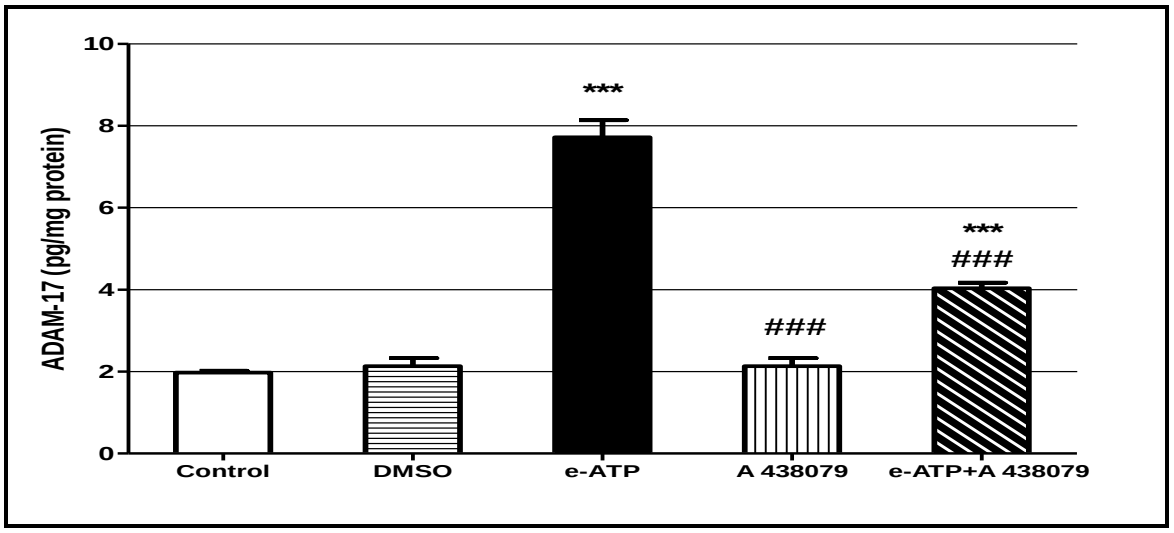

Figure 2. Effect of extracellular ATP or/and P2X7R antagonist (A 438079) on renal ADAM-17.

- Total kidney extracts from rats treated with either saline (control), DMSO, e-ATP (3.3 mg/kg i.p.), P2X7R-specific antagonist A 438079 (3 mg/kg i.p.), or e-ATP in combination with A 438079 for 4 hours were used to measure the ADAM-17 protein levels by ELISA.
- Values are represented as mean ± SEM. (n=6).
- ******* or ### indicates a significant difference from control or e-ATP-alone treated groups respectively at $P<0.001$ using one-way ANOVA tailed by Tukey-Kramer as a *post-hoc* test.
- ADAM-17, a disintegrin and metalloproteinase-17; e-ATP, extracellular adenosine triphosphate.

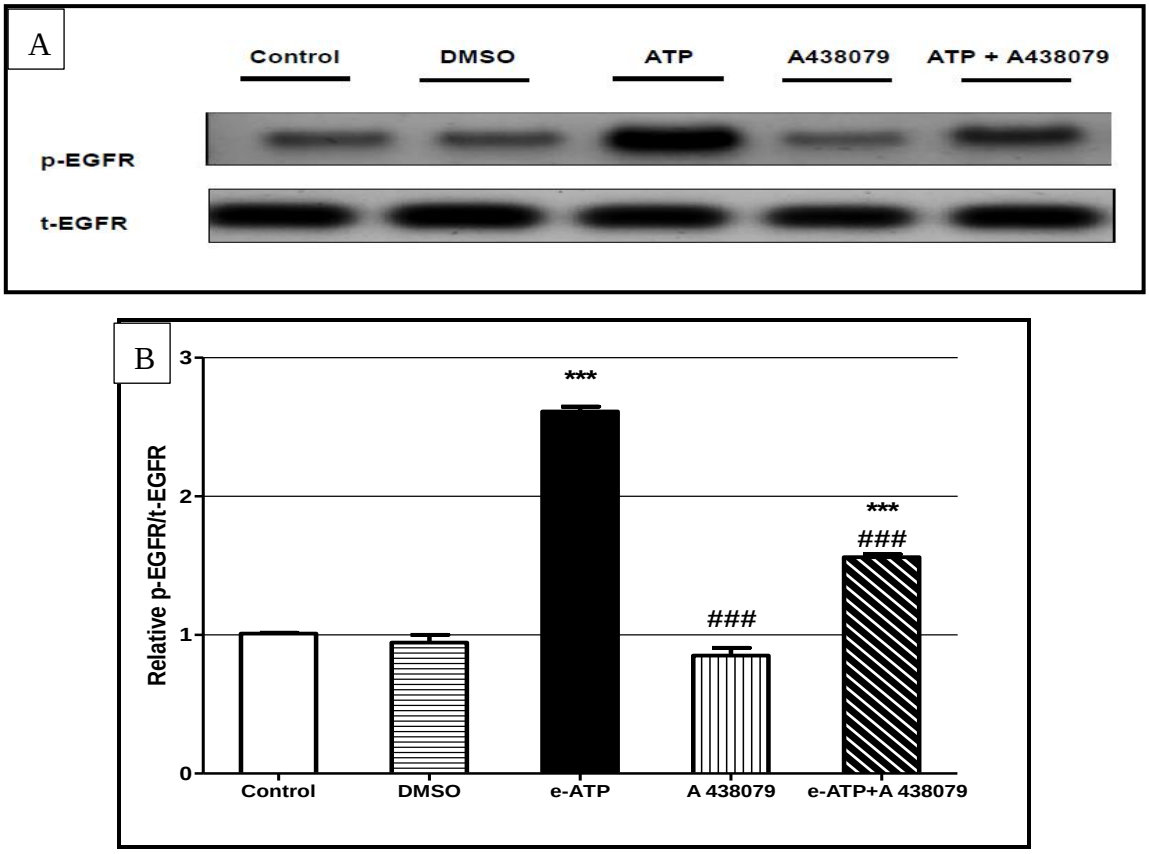

Figure 3. Effect of extracellular ATP or/and P2X7R antagonist (A 438079) on phosphorylation of EGFR in rat kidney.

-Total kidney extracts from rats treated with either saline (control), DMSO, e-ATP (3.3 mg/kg i.p.), P2X7R-specific antagonist A 438079 (3 mg/kg i.p.), or e-ATP in combination with A 438079 for 4 hours were subjected to Western blotting analysis (A) and probed with anti-p-EGFR and total EGFR (t-EGFR) antibodies. The lower panel (B) shows data from densitometric analysis of p-EGFR relative to t-EGFR level.

- Values are represented as mean ± SEM. (n=6).

- ******* or ### indicates a significant difference from control or e-ATP-alone treated groups respectively at P<0.001 using one-way ANOVA tailed by Tukey-Kramer as a *post-hoc* test.

- e-ATP, extracellular adenosine triphosphate; p-EGFR, phosphorylated epidermal growth factor receptor; t-EGFR, total epidermal growth factor receptor.

3.4. Effect of extracellular adenosine triphosphate (e-ATP) or/and the P2X7R antagonist (A 438079) on ERK-1/2 phosphorylation in rat kidney

Obtained results revealed that the ATP-triggered ERK-1/2 phosphorylation was significantly weakened by pretreatment of animals with the P2X7R antagonist, A 438079 (Figure 4). Thus, P2X7Rs are critically involved in ERK-1/2 phosphorylation prompted by ATP.

3.5. Effect of extracellular adenosine triphosphate (e-ATP) or/and the P2X7R antagonist (A 438079) on renal cyclin-B1 mRNA and protein expression

For examination of whether ERK-1/2 phosphorylation induced by ATP could be translated into an increment in the expression of the G2/M regulator, cyclin B1, the renal mRNA, as well as protein expression of cyclin B1, were assessed in ATP-treated rats either in the absence or presence of the P2X7R antagonist, A 438079. Results obtained exhibited a significant increase in cyclin B1 mRNA and protein expression by ATP administration (Figure 5 & 6 respectively). The ATP-induced cyclin B1 mRNA and associated protein expression were significantly prevented in the presence of A 438079 (Figure 5 & 6 respectively).

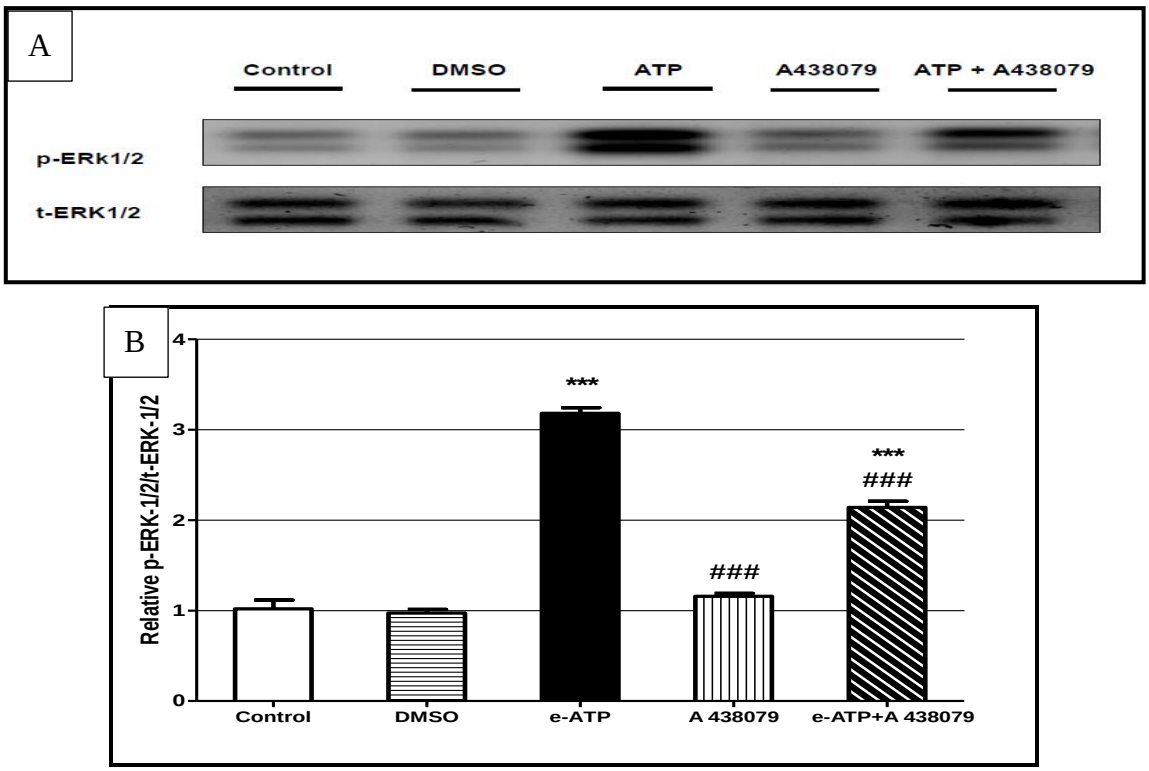

Figure 4. Effect of extracellular ATP or/and P2X7R antagonist (A 438079) on ERK-1/2 phosphorylation in rat kidney.

-Total kidney extracts from rats treated with either saline (control), DMSO, e-ATP (3.3 mg/kg i.p.), P2X7R-specific antagonist A 438079 (3 mg/kg i.p.), or e-ATP in combination with A 438079 for 4 hours were subjected to Western blotting analysis (A) and probed with anti-p-ERK-1/2 and total ERK-1/2 (t-ERK-1/2) antibodies. The lower panel (B) shows data from densitometric analysis of p-ERK-1/2 relative to t-ERK-1/2 level. - Values are represented as mean ± SEM. (n=6).

- ******* or ### indicates a significant difference from control or e-ATP-alone treated groups respectively at P<0.001 using one-way ANOVA tailed by Tukey- Kramer as a *post-hoc* test.

- e-ATP, extracellular adenosine triphosphate; p-ERK-1/2, phosphorylated extracellular signal regulated kinase-1/2; t-ERK-1/2, total extracellular signal regulated kinase-1/2.

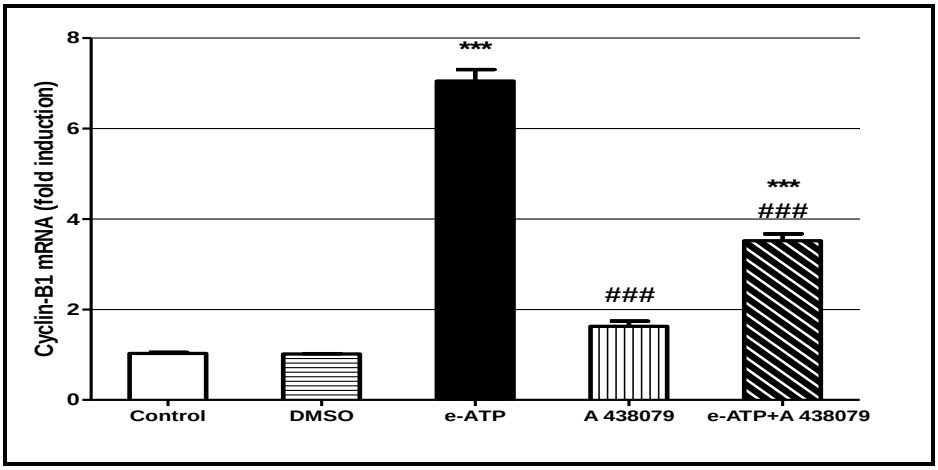

Figure 5. Effect of extracellular ATP or/and P2X7R antagonist (A 438079) on renal cyclin-B1 mRNA expression.

- Total RNA was extracted from kidney tissues of rats treated with either saline (control), DMSO, e-ATP (3.33 mg/kg i.p.), P2X7R-specific antagonist A 438079 (3 mg/kg i.p.), or e-ATP in combination with A 438079 for 24h and mRNA expression of Cyclin-B1 was determined by qRT-PCR analysis. Cyclin-B1 mRNA was normalized to that of GAPDH and is shown as mean fold-induction.- Values are represented as mean ± SEM. (n=6).

- ******* or ### indicates a significant difference from control or e-ATP-alone treated groups respectively at P<0.001 using one-way ANOVA tailed by Tukey-Kramer as a *post-hoc* test.

- e-ATP, extracellular adenosine triphosphate.

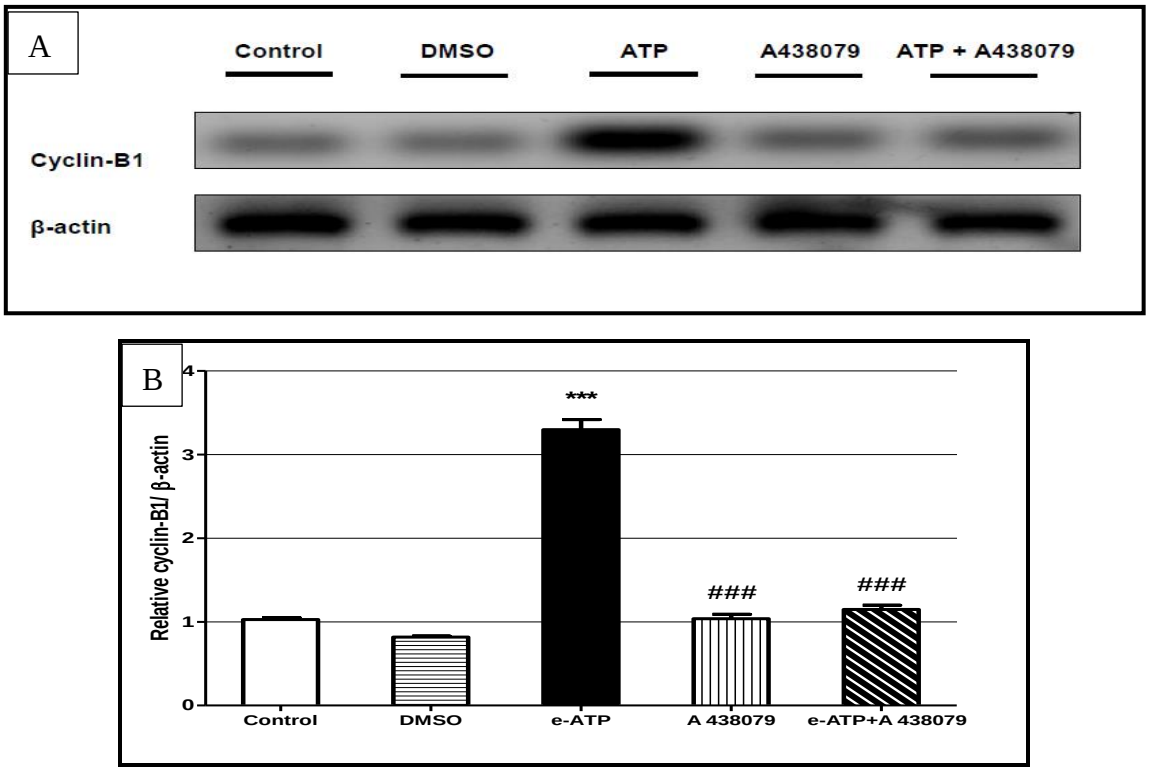

Figure 6. Effect of extracellular ATP or/and P2X7R specific antagonist (A 438079) on renal cyclin-B1 protein expression.
- Total kidney extracts from rats treated with either saline (control), DMSO, e-ATP (3.3 mg/kg i.p.), P2X7R-specific antagonist A 438079 (3 mg/kg i.p.), or e-ATP in combination with A 438079 for 24 h were subjected to Western blotting analysis (A) and probed with anti-cyclin-B1 and β-actin antibodies. The lower panel (B) shows data from densitometric analysis of cyclin-B1 relative to β-actin level. - Values are represented as mean ± SEM. (n=6).
- ******* or ### indicates a significant difference from control or e-ATP-alone treated groups respectively at P<0.001 using one-way ANOVA tailed by Tukey-Kramer as a *post-hoc* test.
- e-ATP, extracellular adenosine triphosphate.

3.6. Effect of extracellular adenosine triphosphate (e-ATP) or/and the P2X7R antagonist (A 438079) on serum level of nitric oxide (NO)

As presented in Figure (7), the serum level of NO was significantly increased by ATP administration, an effect that was markedly repressed in the presence of A 438079. Thus, the P2X7Rs are involved in the increased NO levels induced by ATP.

4. DISCUSSION

For more than 20 years, ATP has been sold as a food supplement. ATP is generally safe and is not involved in the forbidden list of the World Anti-Doping Agency [14]. Previously, it has been reported that ATP activates the classical MAPK/ERK cascade and subsequent cell proliferation of rat renal mesangial cells [15]. Thus, we hypothesized that the safety of ATP supplementation as an ergogenic aid for muscle building and performance enhancement is not absolute. Accordingly, we investigated first, whether the ATP-induced ERK-1/2 signaling cascade could also occur *in vivo*. Second, the potential involvement of P2X7R in ERK-1/2 phosphorylation prompted by ATP was explored using the P2X7R antagonist, A 438079. Results from the current work reveal that ATP administration activates the ERK-1/2 signaling cascade in the rat kidney with a peak measured after 4 h. Noteworthy, administration of A 438079 one hour before ATP significantly reduced ERK-1/2 activation in kidney tissues. In line with these results, a clear reduction in ATP-triggered ERK-1/2 phosphorylation has been verified upon treatment of different cultured cells with different antagonists to the P2X7Rs [16-19].

To investigate the molecular mechanism through which ATP induces ERK-1/2 phosphorylation in renal tissues, the levels of ADAM-17, as well as EGFR and ERK-1/2 phosphorylation in renal tissues, were examined in

the presence of ATP and/or the P2X7R antagonist, A 438079. It was found that ATP could enhance the phosphorylation of EGFR and ERK-1/2 along with the up-regulation of ADAM-17 level. These effects were largely attenuated in animals pre-treated with A 438079. In agreement with these results, **Boots and coworkers** reported that in human bronchial epithelial cells, the ATP-mediated activation of ERK-1/2 and NF-κB pathways was found to be associated with EGFR ligand shedding by ADAM-17 [20].

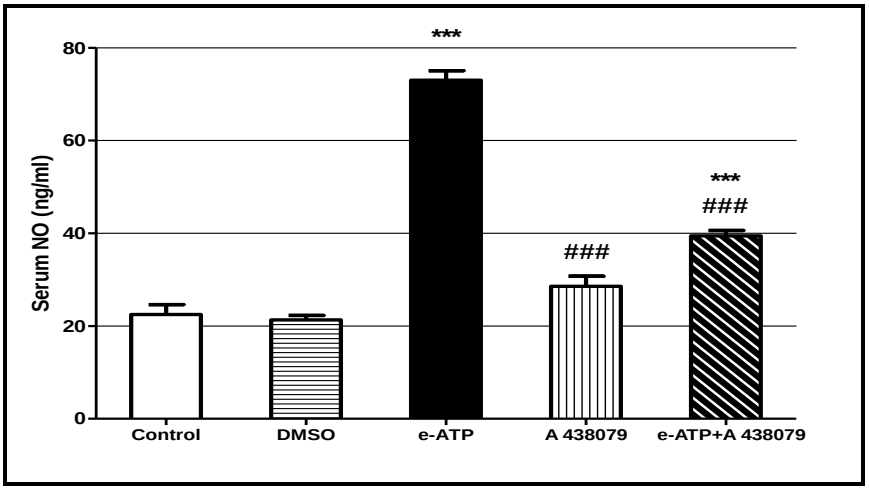

Figure 7. Effect of extracellular ATP or/and P2X7R-specific antagonist (A 438079) on serum NO in rats after 24h.

- Serum levels of NO in rats treated with either saline (control), DMSO, e-ATP (3.3 mg/kg i.p.), P2X7R-specific antagonist A 438079 (3 mg/kg i.p.), or e-ATP in combination with A 438079 for 24 h.
- Values are represented as mean ± SEM. (n=6).
- ******* or ### indicates a significant difference from control or e-ATP-alone treated groups respectively at P<0.001 using one-way ANOVA tailed by Tukey- Kramer as a *post-hoc* test.
- e-ATP, extracellular adenosine triphosphate; NO, nitric oxide.

Besides, our results were mostly supported by a previous study in which the extracellular ADAM-17 activity noticed subsequently after ATP stimulation was reduced in P2X7R deficient macrophages or when A438079; the P2X7R-specific antagonist was incubated with the cells [21]. Extracellular ATP activated P2X7R, raised intracellular calcium, and increased reactive oxygen species (ROS) generation, events that are important for the activation of the metalloproteinase ADAM-17 [21,22]. Extensive data showed that ADAM-17 is accountable for shedding of EGFR ligand, such as pro-HB-EGF, pro-TGF-α, amphiregulin, or epiregulin in various cells [23]. Binding of growth factor to an EGFR activates the mitogenic RAS/RAF/MEK/ERK signaling cascade [24]. In the current work, the mRNA and protein expression of the G2/M regulator, cyclin B1 was enhanced in ATP-treated animals, an effect that was attenuated in presence of A 438079. These results indicate that ATP/P2X7R-induced EGFR/ERK-1/2 signaling activation regulates cell cycle progression by modulating cyclin B1 expression. Earlier, the cyclin B1 expression during G2/M-phase was found to be correlated with ERK activity in synchronized Hela cells [25]. Consistent with our observations, **Chen and coworkers** reported that ATP activates $P2Y_2$ and $P2X_{4/7}$ receptors and enhances the proliferation of human cardiac fibroblasts by inducing cell cycling progression [26].

Nevertheless, what has not been investigated is the implication of the NO in ATP-induced renal injury. Herein, the current study demonstrated that ATP administration showed a significant elevation of the serum NO level, an effect that is diminished in the pre-treatment of animals with the P2X7R antagonist, A438079. In agreement with current results, ATP was found to stimulate the production of NO in the thick ascending limb mainly through activation of P2X receptor [27,28]. Moreover, evidence for P2X7R activation leading to ROS and reactive nitrogen species (RNS) generation has been described in rat microglia, astrocytes RAW 264.7 macrophages as well as in human eosinophil [29-32]. In further support, the ATP-induced generation of H_2O_2 in mononuclear blood cells was inhibited using the specific P2X7R antagonist, AZD9056 [33]. In the present work, the elevated serum level of NO induced by ATP is suggested to be a crucial event

leading to cell proliferation. Indeed, the mitogenic properties of NO have been well documented [34]. In rabbit aortic endothelial cells, the low-molecular-weight *S*-nitrosothiol *S*-nitroso-*N*-acetylpenicillamine induces p21 Ras nitrosation and hence Ras-ERK-1/2-MAPK signaling pathway activation ultimately leading to cell-cycle progression [35]. Moreover, NO was reported as a proliferative agent in human fetal lung fibroblast. The possible molecular mechanisms involved are inhibition of p21 and p27, the activation of cyclin/CDK complexes in addition to hyperphosphorylation of retinoblastoma [36].

5. CONCLUSIONS

The present study evidenced for the first time that ATP, through P2X7Rs, could activate the ERK-1/2 signaling cascade as indicated by an increase in ADAM-17, p-EGFR, pERK-1/2 and subsequent expression of the cell mitogenic gene cyclin B1 in rat kidney. Furthermore, this study presents the mechanistic relevance of RNS in the activation of these signaling cascades. Finally, the results obtained in this experiment provide an argument against the therapeutic usefulness of ATP. Thus, the reported safety of ATP supplements as muscle building and performance enhancers should be reassessed.
Supplementary Materials:

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Conflicts of Interest: None of the authors has conflicts of interest to declare.

Ethical Statement: This experiment was done according to the Ethics Committee of Faculty of Pharmacy (Girls), Al-Azhar University (Approval number: 177/2017) in compliance with the standards of Principles of Laboratory Animal Care (NIH Publications No. 85-23, revised 2011).

Author Contribution: El-Sayed Akool and Amany Balah developed the research idea, designed the experiments, supervised the experiments performance and revised the manuscript. Somaia A. Abdel-Sattar supervised the experiments performance, executed data analysis, wrote and revised the manuscript. Fatma Mounieb performed the experiments, collected the data, carried out the graphical and statistical analysis and wrote the manuscript. The authors declare that all data were generated in-house and that no paper mill was used.

List of Abbreviations: ADAM-17, A disintegrin metalloenzyme-17; ATP, adenosine 5′ triphosphate; DMSO, dimethyl sulfoxide; e-ATP, extracellular adenosine triphosphate; MAPK, mitogen activated protein kinase; NO, nitric oxide; P2X7R, purinergic P2X7 receptor; p-EGFR, phosphorylated epidermal growth factor receptor; p- ERK-1/2, phosphorylated extracellular signal regulated kinase-1/2; t-EGFR, total epidermal growth factor receptor; t- ERK-1/2, total extracellular signal regulated kinase-1/2.

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