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ATP Stimulates Human Placental 11β-Hydroxysteroid Dehydrogenase Type 2 Activity by A Novel Mechanism Independent of Phosphorylation

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Abstract The human placental 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) is believed to play a key role in fetal development since this enzyme protects the fetus from exposure to high levels of maternal cortisol by virtue of converting maternal cortisol to its inert metabolite cortisone. The present study was undertaken to examine the effect of ATP on 11β-HSD2 activity in human placental microsomes. Enzyme activity, reflected by the rate of conversion of cortisol to cortisone, was stimulated more than six-fold by 0.5 mM ATP ($EC_{50} = 0.2$ mM). Such stimulation appears to be mediated through a novel mechanism independent of ATP-induced phosphorylation of the reaction components since AMP-PNP, a non-hydrolyzable analogue of ATP, was equally effective. The ATP-induced stimulation of 11β-HSD2 activity is adenine nucleotide specific in that a similar stimulation was observed with ADP and AMP but not with CTP, GTP, or UTP. Furthermore, ATP increased the maximal velocity (V_{max}) of the 11β-HSD2 catalyzed conversion of cortisol to cortisone without altering the apparent K_m of 11β-HSD2 for cortisol, suggesting that ATP may stimulate enzyme activity by interacting with the enzyme at a site other than that involved in substrate binding. In conclusion, the present study has identified ATP as a novel regulator of human placental 11β-HSD2 in vitro. It is conceivable that intracellular ATP may have a profound effect on 11β-HSD2 function in vivo. J. Cell. Biochem. 84: 295–300, 2002.

Key words: glucocorticoid; enzyme activity; microsome; post-translational regulation; fetal development

The 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) is a microsomal enzyme responsible for converting bioactive glucocorticoids (cortisol and corticosterone) to their inactive metabolites (cortisone and 11-dehydrocorticosterone) [White et al., 1997; Stewart and Krozowski, 1999]. 11β-HSD2 is expressed essentially in aldosterone-target organs such as the kidney, where it has a well-established role in blood pressure regulation via its protection of the non-selective mineralocorticoid receptors from high circulating levels of glucocorticoids [Edwards et al., 1988; Funder et al., 1988]. In the human, 11β-HSD2 is also highly expressed in the placenta throughout pregnancy [Shams et al., 1998].

In the human placenta, 11β-HSD2 is believed to play a key role in fetal development [Pepe and Albrecht, 1995; Seckl, 1997; Yang, 1997]. Given that it is expressed primarily in the syncytiotrophoblast layer [Krozowski et al., 1995; Pepe et al., 2001], the site of fetal-maternal exchange, placental 11β-HSD2 is ideally situated to serve as a functional barrier to protect the fetus from being exposed to high circulating levels of maternal cortisol. It has been documented that excessive exposure to glucocorticoids in utero leads to intrauterine growth restriction (IUGR) [Reinisch et al., 1978; Mosier et al., 1982; Novy and Walsh, 1983]. Furthermore, placental 11β-HSD2 activity is attenuated in pregnancies complicated with IUGR [Shams et al., 1998]. In addition, IUGR is a characteristic feature of the syndrome of apparent mineralocorticoid

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excess in which point mutations of the 11β-HSD2 gene render the enzyme inactive [Kitanaka et al., 1996]. Therefore, the study of placental 11β-HSD2 regulation is essential for our complete understanding of fetal growth and placental function in normal and pathological pregnancies.

To date, the majority of studies on the regulation of placental 11β-HSD2 have been directed at the pre-translational level [Pasquarette et al., 1996; Sun et al., 1997, 1998; Pepe et al., 1999; Tremblay et al., 1999]. Until now, it was unknown whether 11β-HSD2 is subjected to post-translational regulation [Hardy et al., 2001]. Given that phosphorylation is the most common and important mechanism of acute and reversible regulation of protein/enzyme function, the present study was designed to determine whether ATP, an essential component of phosphorylation reactions, regulates placental 11β-HSD2 activity through phosphorylation of the 11β-HSD2 protein. Our results demonstrate that ATP stimulates 11β-HSD2 activity over six-fold in human placental microsomes but does so through a novel mechanism independent of phosphorylation.

MATERIALS AND METHODS

Reagents and Supplies

[1,2,6,7-^{3}H(N)]-Cortisol (80 Ci/mmol) was purchased from Du Pont Canada, Inc. (Markham, Ontario). Non-radioactive steroids were obtained from Steraloids, Inc. (Wilton, NH). Polyester-backed thin-layer chromatography (TLC) plates were obtained from Fisher Scientific Ltd. (Unionville, Ontario). All solvents used were OmniSolve grade from BDH, Inc. (Toronto, Ontario). All other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, Ontario).

Isolation of Placental Microsomes

Subcellular fractions were prepared by a standard procedure as described previously [Hardy et al., 2001]. Briefly, human placental tissues (5–10 g), freshly obtained from normal cesarian section deliveries at term, were homogenized in 5 vol of ice-cold 10 mM Tris buffer (pH 7.4) containing 100 mM KCl and 250 mM sucrose (Buffer A). All subsequent steps were performed at 0–4°C. The homogenates were sedimented consecutively at 750 and 20,000*g* for 30 min. The latter supernatant was sedimented at 105,000*g* for 60 min, and the resulting pellets resuspended in appropriate amounts of Buffer A to give a protein concentration of 5–10 mg/ml. This suspension, taken as the microsomal fraction, was stored in small aliquots at −70°C. Protein concentration was determined by the Bradford method using a Bio-Rad (Mississauga, Ontario) protein assay kit with bovine serum albumin as the standard.

Determination of Microsomal 11β-HSD2 Activity

To assess the direct effect of ATP on placental 11β-HSD2 activity, the microsomes (40–50 μg) were pre-incubated for 6 min at 37°C with various concentrations of ATP (or other chemicals as specified) in a total volume of 50 μl HEPES buffer (50 mM, pH 7.4). At the end of the 6 min pre-incubation, the level of 11β-HSD2 activity was assessed by measuring the rate of conversion of cortisol to cortisone, as described previously [Hardy et al., 2001].

Briefly, the conversion assay was initiated by the addition of the 50 μl pre-incubation mix from the above to 450 μl conversion assay medium. This medium, which had been pre-incubated for 10 min at 37°C, contained 10 mM Tris/100 mM KCl (pH 7.4), approximately 50,000 cpm of the labeled cortisol, and final concentrations of the non-radioactive cortisol and cofactor NAD^+ at 200 nM and 0.5 mM, respectively. The enzymatic reaction was allowed to proceed for 10 min at 37°C (preliminary studies indicated that the rate of reaction was linear with time from 2.5 to 15 min, and the amount of the microsomes between 10–100 μg protein). Blanks containing all the assay components except the microsomes were included in all assays. The reaction was then arrested by rapidly transferring the tubes on ice, and by the addition of 4 ml ethyl acetate containing 40 μg mixture of non-radioactive cortisol and cortisone as carrier steroids. The steroids were extracted, the extracts were dried, and the residues were resuspended. A fraction of the resuspension was spotted on a TLC plate, which was developed in chloroform/methanol (9:1, v/v). The bands containing the labeled cortisol and cortisone were identified by UV illumination of the cold carriers, cut out into scintillation vials and counted in Scintisafe™ Econo 1 (Fisher Scientific, Toronto, Canada). The rate of cortisol to cortisone conversion was then calculated and expressed as the amount of cortisone (pmol)

formed per minute per milligram of protein. Results are shown as mean ± SEM. One-way ANOVA followed by Dunnett's test, and by Tukey's test when necessary, were used to determine statistical differences. Significance was set at $P < 0.05$.

Kinetic analyses were performed as described previously [Hardy et al., 2001]. Conversion assays were conducted using a fixed amount of NAD^+ (0.5 mM), microsomes (40–50 μg protein), and reaction time (10 min), but with varying amounts of cortisol. The conditions were chosen so that the initial velocity was linear with the reaction time and the amount of microsomes. Each experiment was carried out in duplicate, and a total of four independent experiments were conducted. The data were plotted as a straight line of v against v/s according to the Eadie-Hofstee Plot, and the K_m and V_{max} values were calculated from the intercepts of the plots as described [Henderson, 1992].

RESULTS

Effects of ATP and AMP-PNP on Placental 11β-HSD2 Activity

To examine the direct effects of ATP on placental 11β-HSD2 activity, microsomes were prepared from human placentas and incubated with various concentrations of ATP (0.1–1 mM). There was a concentration-dependent increase ($EC_{50} = 0.2$ mM) in the microsomal 11β-HSD2 activity with a maximal effect (over six-fold increase) at 0.5 mM ATP (Fig. 1). As a first step in determining if the ATP-induced stimulation of placental 11β-HSD2 activity was through phosphorylation of reaction components, placental microsomes were incubated with 1 mM AMP-PNP, a non-hydrolyzable analogue of ATP. It was found that AMP-PNP was equally effective in stimulating 11β-HSD2 activity (Fig. 2), indicating that the ATP-induced stimulation was independent of phosphorylation.

Effects of EDTA on the ATP-Induced Stimulation of Placental 11β-HSD2 Activity

Given that ATP can function as a chelator of heavy metals [Lorusso et al., 1990], we then considered the possibility that ATP may stimulate placental 11β-HSD2 activity through chelation of inhibitory heavy metals. To examine this possibility, we studied the effect of EDTA, a well-known metal chelator, on the microsomal

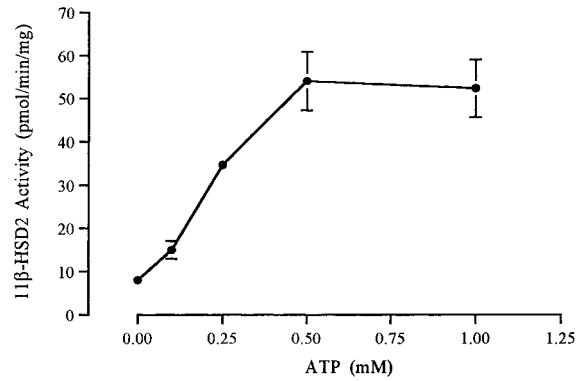

Fig. 1. ATP dose–response curve. Placental microsomes were pre-incubated with increasing concentrations of ATP and were then incubated with cortisol (200 nM) and NAD^+ (0.5 mM) for determining the rate of cortisol to cortisone conversion, as described in Materials and Methods. Each data point represents the mean ± SEM of four independent experiments each performed in duplicate using separate microsomal preparations.

11β-HSD2 activity under basal and ATP-stimulated conditions (Fig. 3). Prior incubation of placental microsomes with 0.5 mM EDTA resulted in a significant increase ($P < 0.05$) in 11β-HSD2 activity. There was no further increase with 1 mM EDTA, indicating that the maximal effect occurred at 0.5 mM EDTA or less. It is noteworthy that both ATP alone and ATP + EDTA resulted in a greater increase ($P < 0.05$) in 11β-HSD2 activity than EDTA alone. However, there was no difference in the stimulation of 11β-HSD2 activity between ATP alone and ATP + EDTA (Fig. 3).

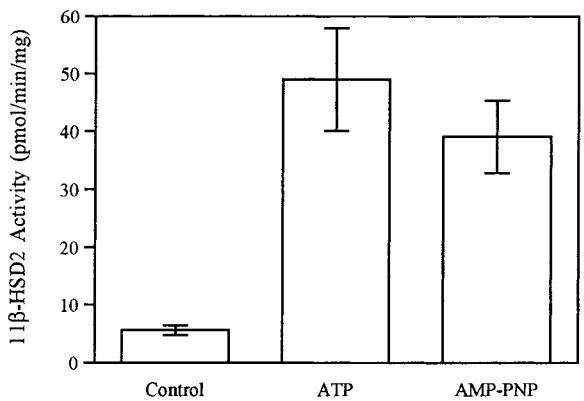

Fig. 2. Effects of AMP–PNP. Placental microsomes were pre-incubated with and without 1 mM ATP or 1 mM AMP-PNP (a non-hydrolyzable analogue of ATP) and were then incubated with cortisol (200 nM) and NAD^+ (0.5 mM) for determining the rate of cortisol to cortisone conversion, as described in Materials and Methods. Each data point represents the mean ± SEM of four independent experiments each performed in duplicate using separate microsomal preparations.

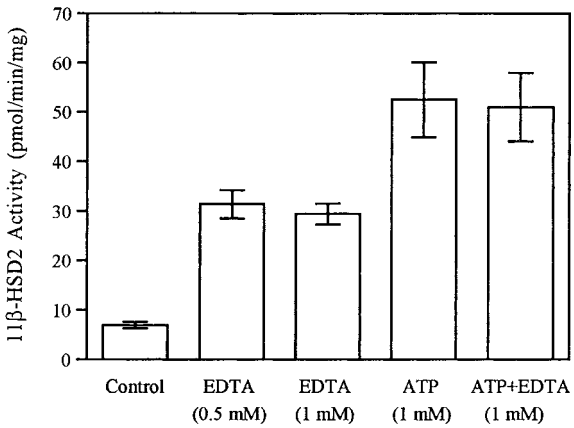

Fig. 3. Effects of EDTA. Placental microsomes were pre-incubated in the absence and presence of ATP with and without EDTA (a well-known chelator of Mg^{2+} and other heavy metals) and were then incubated with cortisol (200 nM) and NAD^+ (0.5 mM) for determining the rate of cortisol to cortisone conversion, as described in Materials and Methods. Each data point represents the mean ± SEM of four independent experiments each performed in duplicate using separate microsomal preparations.

Specificity of the Effects of ATP on the Microsomal 11β-HSD2 Activity

To examine the specificity of the ATP-induced stimulation of 11β-HSD2 activity, placental microsomes were incubated with CTP, GTP, and UTP, respectively. Under conditions of the present study, none of these three nucleotide triphosphates affected the microsomal 11β-HSD2 activity (Fig. 4), indicating that the stimulatory effects of ATP may be adenine nucleotide specific. To examine this further,

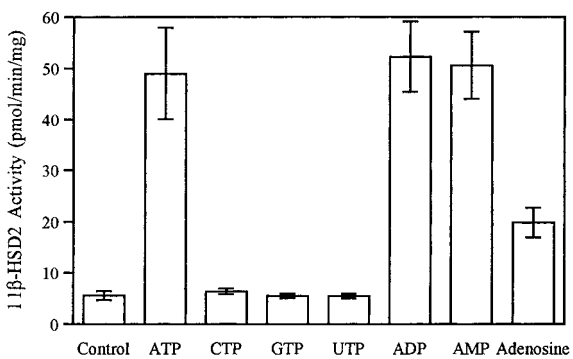

Fig. 4. Specificity of the ATP effects. Placental microsomes were pre-incubated with and without 1 mM of ATP, CTP, GTP, UTP, ADP, AMP, or adenosine and were then incubated with cortisol (200 nM) and NAD^+ (0.5 mM) for determining the rate of cortisol to cortisone conversion, as described in Materials and Methods. Each data point represents the mean ± SEM of four independent experiments each performed in duplicate using separate microsomal preparations.

we determined the effects of ADP, AMP, and adenosine on the microsomal 11β-HSD2 activity. Incubation with ADP or AMP resulted in an increased 11β-HSD2 activity with a similar magnitude to that observed with ATP. Furthermore, adenosine also increased the microsomal 11β-HSD2 activity but to a lesser degree ($P < 0.05$) when compared with ATP, ADP, or AMP (Fig. 4).

Effects of ATP on Kinetic Parameters of Placental 11β-HSD2 Activity

The effects of ATP on kinetic parameters of the microsomal 11β-HSD2 activity were then studied in order to gain insight into the mechanisms underlying the ATP-induced stimulation. ATP at 1 mM increased the V_{max} of 11β-HSD2 catalyzed conversion of cortisol to cortisone without altering the K_m of 11β-HSD2 for cortisol (Table I).

DISCUSSION

In the present study, we have identified ATP as a novel activator of human placental 11β-HSD2 activity. Our results demonstrate that ATP, at physiological concentrations, stimulates 11β-HSD2 activity in human placental microsomes, indicating a direct effect at the post-translational level. Furthermore, the ATP-induced stimulation is adenine nucleotide specific, and does not appear to involve phosphorylation of reaction components or donation of energy to the reaction. In addition, such stimulation is associated with an enhanced catalytic efficiency of 11β-HSD2 catalyzed conversion of cortisol to its inactive metabolite, cortisone. Taken together, these findings suggest a novel role for intracellular ATP in regulating placental 11β-HSD2 activity.

In a previous study, we provided the first evidence that human placental 11β-HSD2 is subject to post-translational regulation by demonstrating that Ca^{2+} inhibited 11β-HSD2 activity in human placental microsomes [Hardy et al., 2001]. Given that phosphorylation is the most common and important mechanism of acute and reversible regulation of protein/enzyme function, in the present study we examined the possibility that placental 11β-HSD2 may be modulated by ATP-induced phosphorylation. Our results demonstrated that ATP, at physiological concentrations, stimulated the microsomal 11β-HSD2 activity more

TABLE I. Effects of ATP on Placental 11β-HSD2 Kinetics

Parameter	V_{max} (pmol cortisone formed/min/mg protein)	K_m for cortisol (nM)
Control	8 ± 1	37 ± 3
ATP (1 mM)	55 ± 9	35 ± 1

Kinetic analyses were performed as described in Materials and Methods, and the K_m and V_{max} values were calculated according to the Eadie-Hofstee Plot. Each data point represents the mean ± SEM of four independent experiments, each performed in duplicate using separate microsomal preparations.

than six-fold. However, the same degree of stimulation was achieved with AMP-PNP, a non-hydrolyzable analogue of ATP, which cannot serve as a phosphoryl donor in phosphotransferase reactions. Moreover, stimulation of enzyme activity by ATP persisted when potential phosphotransferase activity was blocked by chelation of Mg^{2+} with EDTA. Taken together, these data indicate that the stimulatory effect of ATP on placental 11β-HSD2 activity is mediated by a novel mechanism which does not involve phosphorylation or donation of energy.

To gain insight into the mechanisms underlying the ATP-induced stimulation of 11β-HSD2 activity, we investigated the possibility that ATP stimulated the microsomal 11β-HSD2 activity through chelation of inhibitory heavy metals since ATP can function as a metal chelator, and heavy metals are known to inhibit the activity of other enzyme systems [Lorusso et al., 1990]. We first determined whether chelation of heavy metals by EDTA, a well-known chelator, was associated with an enhanced 11β-HSD2 activity. Our results showed that EDTA increased the microsomal 11β-HSD2 activity by more than three-fold. This indicated that certain heavy metals exert inhibitory effects on placental 11β-HSD2 activity in vitro. Indeed, ongoing studies in our laboratory have provided evidence that Zn^{2+} inhibits placental 11β-HSD2 activity (Niu and Yang, unpublished communication). However, the maximal stimulation by EDTA was consistently less than that with ATP, arguing against the notion that ATP-stimulated enzyme activity by chelating inhibitory heavy metals. We reasoned that if the ATP-induced stimulation was independent of chelation, the combined effects of ATP and EDTA should be at least additive. Surprisingly, the increase in the microsomal 11β-HSD2 activity following the combined treatment was similar to that with ATP alone, suggesting that the stimulatory actions of ATP may be mediated by at least two mechanisms: chelation of inhibitory heavy metals and a yet unidentified novel mechanism.

We then examined the specificity of the ATP-induced stimulation by investigating the potential involvement of the nucleotide moiety. Using a concentration greater than that required for the maximal stimulation with ATP, we found that while both ADP and AMP were as effective as ATP in stimulating the microsomal 11β-HSD2 activity, none of the other three nucleotide triphosphates (CTP, GTP, and UTP) affected enzyme activity. This indicated that the ATP-induced stimulation of 11β-HSD2 activity was adenine nucleotide specific. It is noteworthy that adenosine also stimulated 11β-HSD2 activity, but to a lesser degree, indicating that at least one phosphate at the 5′-position of the ribose moiety was required for the maximal stimulation.

Analysis of the influence of ATP on the kinetic parameters of enzyme activity provided further insight into the mechanisms underlying 11β-HSD2 stimulation. Our results demonstrated that the stimulatory action of ATP was associated with an increase in the V_{max} of 11β-HSD2-catalyzed conversion of cortisol to cortisone without changes in the apparent K_m of 11β-HSD2 for cortisol. This suggested that ATP influenced 11β-HSD2 activity at a site other than that involved in the substrate binding. Alternatively, ATP may stimulate enzyme activity indirectly by altering the function of another protein/enzyme. Further studies using either the recombinant protein or the purified enzyme will be required to elucidate the precise mechanisms underlying the ATP-induced stimulation of human placental 11β-HSD2.

Whatever mechanisms of its actions, ATP at physiological concentrations, stimulated placental 11β-HSD2 activity more than six-fold in vitro. This level of stimulation by ATP is far greater than those produced by other known stimulators of placental 11β-HSD2, such as forskolin [Pasquarette et al., 1996] and retinoic acid [Tremblay et al., 1999]. Given that the bulk

of 11β-HSD2 protein including the catalytic domain is on the cytoplasmic side [Obeyesekere et al., 1997; Odermatt et al., 1999] and thus exposed to ATP, it is conceivable that intracellular ATP may have a profound effect on placental 11β-HSD2 function in vivo.

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