TITLE PAGE

The Extracellular cAMP-Adenosine Pathway Significantly Contributes To The *In Vivo* Production Of Adenosine

Edwin K. Jackson, Zaichuan Mi and Raghvendra K. Dubey

Center for Clinical Pharmacology, Departments of Pharmacology (EKJ) and Medicine (ZM, RKD), University of Pittsburgh School of Medicine, Pittsburgh, PA

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B) Corresponding Author:

Edwin K. Jackson, Ph.D.

Center for Clinical Pharmacology, University of Pittsburgh School of Medicine

100 Technology Drive, Suite 450, Pittsburgh, PA 15219-3130

phone: 412-648-1505; fax: 412-648-1837; email: edj@pitt.edu

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D) Abbreviations: 1,3-dipropyl-8-p-sulfophenylxanthine, DPSPX; erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride, EHNA; high pressure liquid chromatography, HPLC; mean arterial blood pressure, MABP; renal blood flow, RBF; glomerular filtration rate, GFR; spontaneously hypertensive rat, SHR; Wistar-Kyoto rat, WKY; analysis of variance, ANOVA; Fisher's Least Significant Difference test, Fisher's LSD.

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ABSTRACT

The extracellular cAMP-adenosine pathway is the cellular egress of cAMP followed by extracellular conversion of cAMP to adenosine by the sequential actions of ecto-phosphodiesterase and ecto-5'-nucleotidase. Although detailed studies in isolated organs, tissues and cells provide evidence for an extracellular cAMP-adenosine pathway, whether this mechanism contributes significantly to adenosine production *in vivo* is unclear. 1,3-Dipropyl-8-p-sulfophenylxanthine is restricted to the extracellular compartment due to a negative charge at physiological pH, and at high concentrations ($\geq$0.1 mmoles/L) blocks ecto-phosphodiesterase. Here we show that administration of 1,3-dipropyl-8-p-sulfophenylxanthine at a dose that provided concentrations in plasma and urine of approximately 0.3 mmoles/L and 6 mmoles/L, respectively, inhibited urinary adenosine excretion. In Sprague-Dawley rats, intravenous 1,3-dipropyl-8-p-sulfophenylxanthine (10 mg + 0.15 mg/min) significantly decreased by 48% and 39% the urinary excretion of adenosine (from 3.57 ± 0.38 to 1.87 ± 0.14 nmoles/30 min; P=0.0003) and the ratio of urinary adenosine to cAMP (from 0.93 ± 0.08 to 0.57 ± 0.06; P=0.0044), respectively, without altering blood pressure, renal blood flow or glomerular filtration rate. Although 1,3-dipropyl-8-p-sulfophenylxanthine transiently increased urine volume and sodium excretion, these effects subsided, yet adenosine excretion remained reduced. Thus changes in systemic and renal hemodynamics and excretory function could not account for the effects of 1,3-dipropyl-8-p-sulfophenylxanthine on adenosine excretion. Additional experiments showed that 1,3-dipropyl-8-p-sulfophenylxanthine, as in Sprague-Dawley rats, significantly attenuated adenosine excretion and the ratio of urinary adenosine to cAMP in both Wistar-Kyoto rats and spontaneously hypertensive rats. We conclude that the extracellular

cAMP-adenosine pathway significantly contributes to the *in vivo* production of adenosine.

INTRODUCTION

Transport of intracellular cAMP into the extracellular compartment appears to be a ubiquitous process. For example, studies document this mechanism in liver, superior cervical ganglia, fibroblasts, glioma cells, heart, adipose tissue and adipocytes (Jackson and Dubey, 2001). The rate of cAMP efflux is proportional to the intracellular levels of cAMP, the process begins within minutes following stimulation of adenylyl cyclase, and the mechanism is energy-dependent and temperature sensitive (Jackson and Dubey, 2001).

The function of extracellular cAMP may be to provide adenosine for activation of adenosine cell-surface receptors. In the interstitial compartment, extracellular AMP is rapidly dephosphorylated by ecto-5'-nucleotidase, an ubiquitous enzyme attached to the cell membrane by a lipid-sugar linkage (Pearson et al., 1985; Misumi et al., 1990; Zimmermann, 1992). Therefore, if ecto-phosphodiesterase is expressed on the cell surface, hormonal activation of adenylyl cyclase would result in extracellular production of adenosine by metabolism of extracellular cAMP to extracellular AMP by ecto-phosphodiesterase, followed by metabolism of extracellular AMP to adenosine by ecto-5'-nucleotidase. This sequence of reactions is called the extracellular cyclic AMP-adenosine pathway. Inasmuch as adenosine would be synthesized in the unstirred water layer by spatially-linked, cell-surface proteins, small increases in cAMP may produce biologically-active concentrations of adenosine in the biophase of cell-surface adenosine receptors, thus promoting autocrine and paracrine effects of adenosine.

Much ***in vitro*** data support the existence of the extracellular cAMP-adenosine pathway. In aortic vascular smooth muscle cells (Dubey et al., 1996) and cardiac fibroblasts (Dubey et al., 2000) in culture, exogenous cAMP is converted to AMP, adenosine and inosine in a

concentration- and time-dependent fashion. Significant increases in extracellular adenosine levels occur with concentrations of cAMP in the medium as low as 1 μmole/L, and steady-state levels of adenosine are achieved in the culture medium within five minutes after adding exogenous cAMP. In addition to aortic vascular smooth muscle cells and cardiac fibroblasts, the extracellular cAMP-adenosine pathway is present in hepatocytes (Gorin and Brenner, 1976; Smoake et al., 1981), neurons (Rosenberg and Dichter, 1989; Rosenberg et al., 1994; Rosenberg and Li, 1995, 1996; Brundege et al., 1997), adipocytes (Kather, 1990; Zacher and Carey, 1999), isolated perfused kidneys (Mi and Jackson, 1996, 1998), cerebral blood vessels (Hong et al., 1999), renal microvessels (Jackson and Mi, 2000), collecting ducts (Jackson et al., 2003), and proximal tubules (Jackson et al., 2006). However, whether the extracellular cAMP-adenosine pathway accounts for a significant portion of adenosine biosynthesis *in vivo* is unknown and is the focus of the present investigation.

METHODS

Animals. Studies utilized adult (14-16 weeks-of-age) male Sprague-Dawley rats, spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) obtained from Taconic Farms (Germantown, NY). The Institutional Animal Care and Use Committee approved all procedures.

Protocol 1. Sprague-Dawley rats (n=16) were anesthetized with Inactin (100 mg/kg, i.p.) and placed on a Deltaphase isothermal pad (Braintree Scientific, Braintree, MA). Body temperature was monitored with a rectal temperature probe (Physitemp Instruments, Clifton, NJ) and maintained at 37 ± 0.5°C by adjusting a heat lamp positioned above the rat. A short section of polyethylene (PE) tubing (PE-240) was placed in the trachea to facilitate respiration. Two PE-50 cannulas were inserted into the right jugular vein and infusions of saline were initiated at 25 μl/min in each cannula. A PE-50 catheter was placed in the left carotid artery for blood sample collection and for measurement of mean arterial blood pressure (MABP) via a digital blood pressure analyzer (model BPA, Micro-Med Inc.). A PE-10 catheter was inserted into the left ureter for urine collection, and a flow probe (model 1RB; Transonic Systems, Inc., Ithaca, NY) was placed on the left renal artery and connected to a transit time flowmeter (model T206; Transonic System, Inc.) for determination of renal blood flow (RBF). A bolus and infusion of inulin [carboxyl-^{14}C] (0.5 μCi bolus followed by 0.035 μCi/min infusion) was administered via one jugular cannula. Also, a bolus (10 mg) and infusion (0.15 mg/min) of 1,3-dipropyl-8-p-sulfophenylxanthine (DPSPX; Sigma-Aldrich, St. Louis, MO) was administered via a separate jugular cannula in half (n=8) of the animals (the other half (n=8) continued to receive saline only; random assignment). DPSPX was rendered highly soluable in saline by titrating with

sodium carbonate to convert the free acid to the sodium salt of DPSPX. DPSPX is restricted to the extracellular compartment due to a negative charge at physiological pH (Tofovic et al., 1991), and at high concentrations (equal to or greater than 0.1 mmoles/L) blocks ecto-phosphodiesterase activity (Dubey et al., 1996, 2000). An intravenous bolus of erythro-9-(2-hydroxy-3-nonyl) adenine hydrochloride (EHNA; 3 mg; Sigma-Aldrich) was administered to all animals to inhibit adenosine deaminase so as to enhance the ability to detect changes in adenosine production. After a 90 minute rest period, parameters were recorded in all animals during three 30-minute renal clearance periods. A mid-point blood sample (0.3 ml) for measurement of radioactivity was collected. Plasma and urine ^{14}C-inulin radioactivity were measured, and renal clearance of ^{14}C-inulin was calculated for estimation of glomerular filtration rate (GFR). Urinary adenosine and cAMP were measured by high pressure liquid chromatography (HPLC) with fluorescence detection as previously described (Jackson et al., 1996). Also, plasma levels of DPSPX were measured in a subset of rats using HPLC with ultraviolet detection as previously described (Tofovic et al., 1991). Urinary sodium and potassium were measured by flame photometery (Instrumentation Laboratory, Lexington, MA).

Protocol 2. SHR (n=17) and WKY (n=16) rats were prepared as described above with the exception that the animals were not given radiolabeled inulin and urinary electrolytes were not measured. As with the first protocol, the animals received an intravenous injection of EHNA (3 mg). After a 60-minute rest period, urine was collected for 20 minutes and hemodynamic parameters were measured and recorded. Next, a bolus (10 mg) and infusion of DPSPX (0.15 mg/min) was administered to 8 WKY and 9 SHR, whereas the other WKY and SHR continued to receive saline only (random assignment).

Statistics. Data were analyzed by repeated measures 2-factor analysis of variance (ANOVA) followed by a Fisher's Least Significant Difference test (Fisher's LSD) if the interaction term in the analysis of variance was significant. The criterion of significance was $p<0.05$. All values in text and figures are means ± SEM.

RESULTS

As shown in Figure 1, in Sprague-Dawley rats that received DPSPX (n=8), the urinary excretion rate of adenosine was significantly (P=0.0003; main effects term in ANOVA) reduced by approximately 48% compared with control rats (n=8). DPSPX did not reduce urinary cAMP excretion, but did significantly (P=0.0044; interaction term in ANOVA) reduce the ratio of urinary adenosine to urinary cAMP excretion (by approximately 39%). In contrast to the effects of DPSPX on urinary adenosine excretion, DPSPX did not affect MABP, RBF or GFR (Figure 2). There was a significant and time-dependent effect of DPSPX on urine volume and sodium excretion (Figure 3; P=0.0305 and P=0.0149 for urine volume and sodium excretion, respectively; interaction term in ANOVA). In this regard, during the first two experimental periods, DPSPX approximately doubled urine volume and sodium excretion (P<0.05; Fisher's LSD); however, this robust diuretic and natriuretic effect waned with time so that during the last experimental period, urine volume and sodium excretion were similar in control versus DPSPX-treated animals. Potassium excretion was not affected by DPSPX during any experimental period. Plasma and urine levels of DPSPX were measured in four Sprague-Dawley rats and were 0.3 ± 0.1 and 6.2 ± 1.5 mmoles/L, respectively.

In the first protocol, DPSPX was given from the outset of the protocol. Therefore, the study design was a parallel control design rather than a paired study design. The purpose of the second study was to confirm the result of the first protocol using a paired design and to extend the results to other strains of rats, i.e., SHR and WKY. As shown in Figure 4, DPSPX was associated with an approximate 56% (P<0.05; Fisher's LSD) and 33% (P<0.05; Fisher's LSD) reduction in urinary adenosine excretion in WKY (n=8) and SHR (n=9), respectively (P<0.0001

and P=0.0057 for WKY and SHR, respectively; interaction term in ANOVA). Urinary adenosine excretion was slightly but significantly ($P<0.05$; Fisher's LSD) increased between the first and second experimental periods in the time-control WKY (n=8), but was not significantly changed in the time-control SHR (n=8). As in the first protocol, DPSPX did not affect urinary cAMP excretion in either WKY (2.29 ± 0.15 and 1.94 ± 0.14 nmoles/20 min before and after DPSPX, respectively) or SHR (2.44 ± 0.11 and 2.92 ± 0.16 nmoles/20 min before and after DPSPX, respectively). As shown in Figure 5, DPSPX was associated with an approximate 48% ($P<0.05$; Fisher's LSD) and 44% ($P<0.05$; Fisher's LSD) reduction in the urinary adenosine to urinary cAMP excretion ratio in WKY and SHR, respectively ($P=0.0075$ and $P=0.0035$ for WKY and SHR, respectively; interaction term in ANOVA). Urinary adenosine to urinary cAMP excretion ratios were not significantly changed in the time-control WKY or SHR. As in Sprague-Dawley rats, DPSPX increased urine volume in both WKY and SHR (in WKY, 194 ± 93 and 461 ± 99 μl/20 min before and after DPSPX, respectively, $P<0.05$, Fisher's LSD; in SHR, 260 ± 34 and 358 ± 56 μl/min before and after DPSPX, respectively, $P<0.05$, Fisher's LSD). Also, as in Sprague-Dawley rats, DPSPX did not affect MABP or RBF in either WKY or SHR (data not shown).

DISCUSSION

The goal of the present study was to test the hypothesis that metabolism of extracellular, endogenous cAMP contributes to adenosine formation *in vivo*. To test this hypothesis we measured urinary adenosine as an index of adenosine production and treated animals intravenously with DPSPX to block ecto-phosphodiesterase, the enzyme that converts extracellular cAMP to AMP (which in turn is metabolized to adenosine by ecto-5'-nucleotidase). We used urinary adenosine as a marker for total body adenosine production for two reasons. First, under normal physiological conditions, urinary adenosine is derived in part from filtered adenosine that escapes uptake and metabolism by tubular epithelial cells and in part from renal tubular production of adenosine (Thompson et al., 1985; Heyne et al., 2004). Second, plasma, but not urinary, adenosine measurements are fraught with technical problems that give rise to misleading results because of rapid uptake and metabolism by cells in whole blood and because of rapid synthesis of adenosine in whole blood. Because urine does not normally contain enzymes of adenosine production or metabolism, measurement of urinary adenosine should provide a more reliable index of adenosine production.

A key feature of this experimental strategy was the use of DPSPX. DPSPX is a xanthine that is restricted to the extracellular compartment due to a negative charge at physiological pH (Tofovic et al., 1991). At low concentrations, DPSPX blocks adenosine receptors (Daly and Jacobson, 1995), but at high concentrations DPSPX inhibits ecto-phosphodiesterase (Zacher and Carey, 1999). For example, DPSPX at concentrations greater than or equal to 0.1 mmoles/L inhibits the metabolism of extracellular cAMP to AMP in the isolated perfused kidney (Mi and Jackson, 1995), cultured aortic vascular smooth muscle cells (Dubey et al., 1996), cultured

cardiac fibroblasts (Dubey et al., 2000), freshly isolated preglomerular vascular smooth muscle cells (Jackson and Mi, 2000), cultured oviduct cells (Cometti et al., 2003), freshly isolated collecting ducts and cultured collecting duct cells (Jackson et al., 2003) and freshly isolated proximal tubules and cultured collecting duct cells (Jackson et al., 2006).

In the present study, we administered a bolus of DPSPX of 10 mg followed by an infusion of 0.15 mg/min. This experimental paradigm provided plasma levels of DPSPX, as measured by HPLC with ultraviolet detection, of approximately 0.3 mmoles/L, a concentration well within the range to inhibit ecto-phosphodiesterase. Notably, we achieved urinary concentrations of DPSPX of approximately 6 mmoles/L, 60-fold above the concentration required to inhibit ecto-phosphodiesterase. The high urinary concentrations of DPSPX are likely due to the fact that DPSPX does not cross cell membranes and therefore is concentrated in the urinary compartment as water is reabsorbed from the tubules. Therefore, DPSPX should afford a high degree of inhibition of ecto-phosphodiesterase in the renal tubular system.

It is unlikely that the ability of DPSPX to reduce the urinary excretion of adenosine was secondary to changes in systemic hemodynamics, renal hemodynamics, glomerular filtration rate or renal excretory function. In the present study we did not detect an effect of DPSPX on arterial blood pressure, renal perfusion or glomerular filtration. Because DPSPX is an adenosine receptor blocker (Daley and Jacobson, 1996) and because blockade of A_1 adenosine receptors reproducibly increases urine volume and sodium excretion (Kuan et al., 1993), it is not surprising that in the current study DPSPX caused a large initial increase in urine volume and sodium excretion. However, as has been noted previously, diuretic breaking occurs quickly following A_1-receptor blockade (Bak and Thomsen, 2004), and therefore with time we observed

a gradual return of urine volume and sodium excretion to basal values. Despite the return of urine volume and sodium excretion to baseline levels the excretion of adenosine remained attenuated in DPSPX-treated animals. This observation would appear to rule-out the possibility that the effects of DPSPX on adenosine excretion were mediated by the transient changes in urine volume and sodium excretion. Also, the fact that DPSPX did not significantly affect cAMP excretion but did reduce the ratio of adenosine to cAMP excretion argues strongly that the effects of DPSPX were not merely secondary to changes in urine volume or renal excretory function. Consistent with this conclusion, Heyne et al. (2004) report that urine volume and sodium excretion have little if any effect on the urinary excretion of adenosine.

Currently, DPSPX is the only know inhibitor of ecto-phosphodiesterase that is restricted to the extracellular compartment, and therefore was the preferred inhibitor for the present study. However, as mentioned, DPSPX is also an adenosine receptor antagonist, and therefore it is conceivable that the reduction in urinary adenosine excretion was related to blockade of adenosine receptors. However this seems unlikely because blockade of A_1 receptors increases, rather than decreases, extracellular levels of adenosine (Andresen et al.,1999). Although DPSPX decreases urinary adenosine excretion it does not increase urinary cAMP excretion. This could be due to the fact that by blocking A_2 receptors and decreasing adenosine levels, DPSPX inhibits A_2 receptor-mediated activation of adenylyl cyclase.

The results of the present study are in accord with our previously published work using 3-isobutyl-1-methylxanthine (IBMX) (Mi et al., 1994), an inhibitor of both intracellular and extracellular phosphodiesterase. When added to the perfusate of a microdialysis probe inserted into the renal cortex of anesthetized rats, IBMX reduced the recovery of adenosine and inosine

(metabolite of adenosine) by about 40 to 50%, which is very similar to the reduction in urinary adenosine achieved in the present study. However, because IBMX enters cells and inhibits intracellular as well as extracellular phosphodiesterase, our previous study left open the possibility that metabolism of cAMP to adenosine intracellularly was responsible for the cAMP-adenosine pathway, rather than extracellular cAMP-adenosine pathway. Taken together, the two studies support the concept that the extracellular cAMP-adenosine pathway importantly contributes to adenosine production *in vivo*.

DPSPX reduces urinary adenosine excretion in Sprague-Dawley rats, SHR and WKY. This indicates that the cAMP-adenosine pathway contributes to the *in vivo* production of adenosine in several different strains of rats. An unexpected finding of the present study was the fact that basal urinary adenosine excretion in SHR is approximately 50% of the basal urinary adenosine excretion in WKY or Sprague-Dawley rats. We do not know the mechanism of this potentially important difference between genetically hypertensive and normotensive rats. However, given the importance of adenosine in modulating the growth of cardiac fibroblasts (Dubey et al., 1997), vascular smooth muscle cells (Dubey et al., 1998)) and mesangial cells (Dubey et al., 2005), it is conceivable that reduced levels of adenosine could participate in the pathophysiology of genetic hypertension or its sequelae.

Biochemical and pharmacological evidence strongly supports the existence of the cAMP-adenosine pathway *in vitro*, and the present study suggests that the cAMP-adenosine pathway also exits *in vivo*. In addition to biochemical and pharmacological evidence supporting the cAMP-adenosine pathway, molecular characterization of the components of the system is progressing. In this regard, the molecular details of one component of the pathway, i.e., the

ecto-5'-nucleotidase mediating the metabolism of AMP to adenosine, are well described (Zimmermann, 1992), and molecular studies identify several cAMP transporters that may participate in the efflux of cAMP to the extracellular compartment (Sekine et al., 1997; van Aubel et al., 2002). However, the molecular identity of the ecto-phosphodiesterase that mediates the extracellular metabolism of cAMP to AMP remains unclear. With regard to classical phosphodiesterases, in mammals there are at least 11 gene families, and each family is composed of 1 to 4 distinct genes. Moreover, many of these genes give rise to splice variants so that mammals produce more than 50 different phosphodiesterase proteins (Lugnier, 2006). In addition, mammalian cells express at least three different functional ecto-nucleotide pyrophosphatases (E-NPPs), and these cell-surface enzymes are capable of hydrolyzing cAMP to AMP (Goding et al., 2003). It is conceivable that ecto-phosphodiesterase is comprised of one or more of the aforementioned enzymes or is a yet-to-be-discovered enzyme distinct from classical phosphodiesterases or E-NPPs. Further work is needed to identify the precise molecular identity of ecto-phosphodiesterase.

In summary, the present study demonstrates that systemic administration of DPSPX achieves high levels of DPSPX in plasma and more notably in urine and this is associated with a substantial decrease in the urinary excretion of adenosine. We conclude that the extracellular cAMP-adenosine pathway importantly contributes to adenosine production *in vivo*.

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FOOTNOTES

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LEGENDS FOR FIGURES

Figure 1: Urinary excretion rate of adenosine (top panel) and cAMP (middle panel) and the ratio of adenosine to cAMP excretion in the urine (bottom panel) in control Sprague-Dawley rats (n=8; solid bars) and Sprague-Dawley rats pretreated with DPSPX (10 mg bolus plus 0.15 mg/min infusion; n=8; hatched bars) during three back-to-back renal clearance periods. $^{a}P<0.05$, Fisher's LSD. Data are shown as means ± SEM.

Figure 2: MABP (top panel), RBF (middle panel) and GFR (bottom panel) in control Sprague-Dawley rats (n=8; solid bars) and Sprague-Dawley rats pretreated with DPSPX (10 mg bolus plus 0.15 mg/min infusion; n=8; hatched bars) during three back-to-back renal clearance periods. Data are shown as means ± SEM.

Figure 3: Urine volume (top panel), sodium excretion (middle panel) and potassium excretion (bottom panel) in control Sprague-Dawley rats (n=8; solid bars) and Sprague-Dawley rats pretreated with DPSPX (10 mg bolus plus 0.15 mg/min infusion; n=8; hatched bars) during three back-to-back renal clearance periods. $^{a}P<0.05$, Fisher's LSD. Data are shown as means ± SEM.

Figure 4: Urinary excretion of adenosine during two experimental periods in WKY (top panels) and SHR (bottom panels). WKY-Saline (n=8) and SHR-Saline (n=8) groups received only

saline between experimental periods 1 and 2. WKY-DPSPX (n=8) and SHR-DPSPX (n=9) groups received DPSPX (10 mg bolus plus 0.15 mg/min) between experimental periods 1 and 2. [a]P<0.05, Fisher's LSD. Data are shown as means ± SEM.

Figure 5: Adenosine to cAMP urinary excretion ratios during two experimental periods in WKY (top panels) and SHR (bottom panels). WKY-Saline (n=8) and SHR-Saline (n=8) groups received only saline between experimental periods 1 and 2. WKY-DPSPX (n=8) and SHR-DPSPX (n=9) groups received DPSPX (10 mg bolus plus 0.15 mg/min) between experimental periods 1 and 2. [a]P<0.05, Fisher's LSD. Data are shown as means ± SEM.

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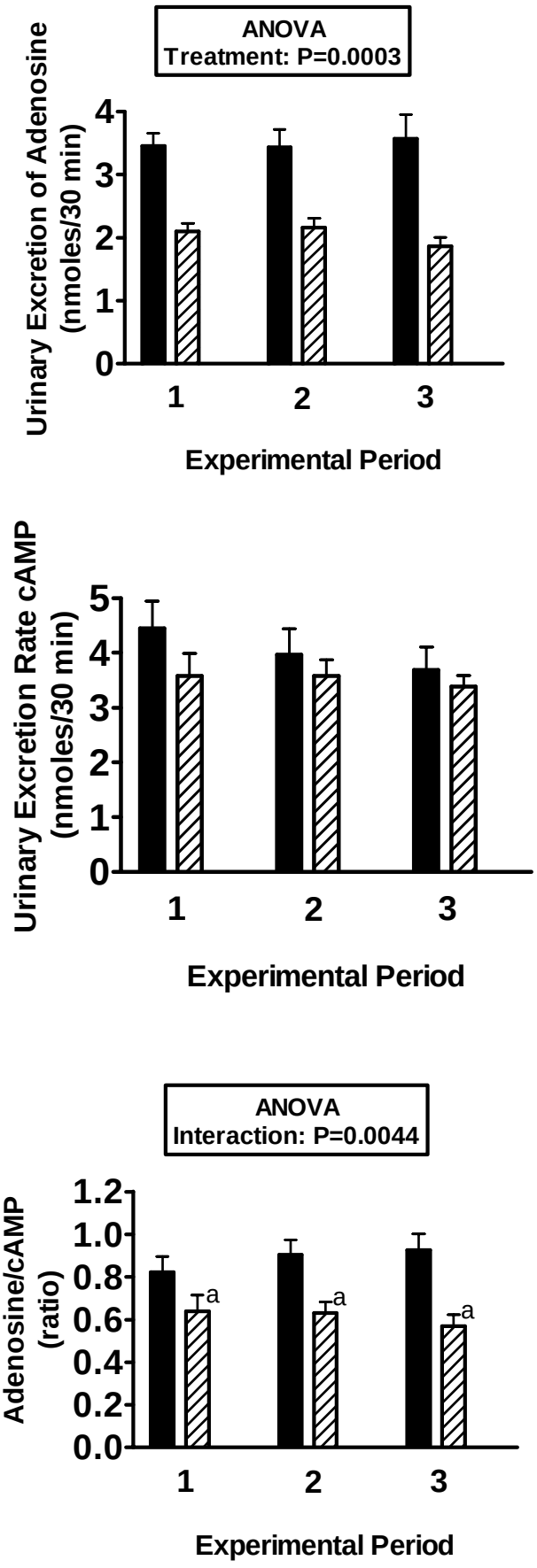

Figure 1

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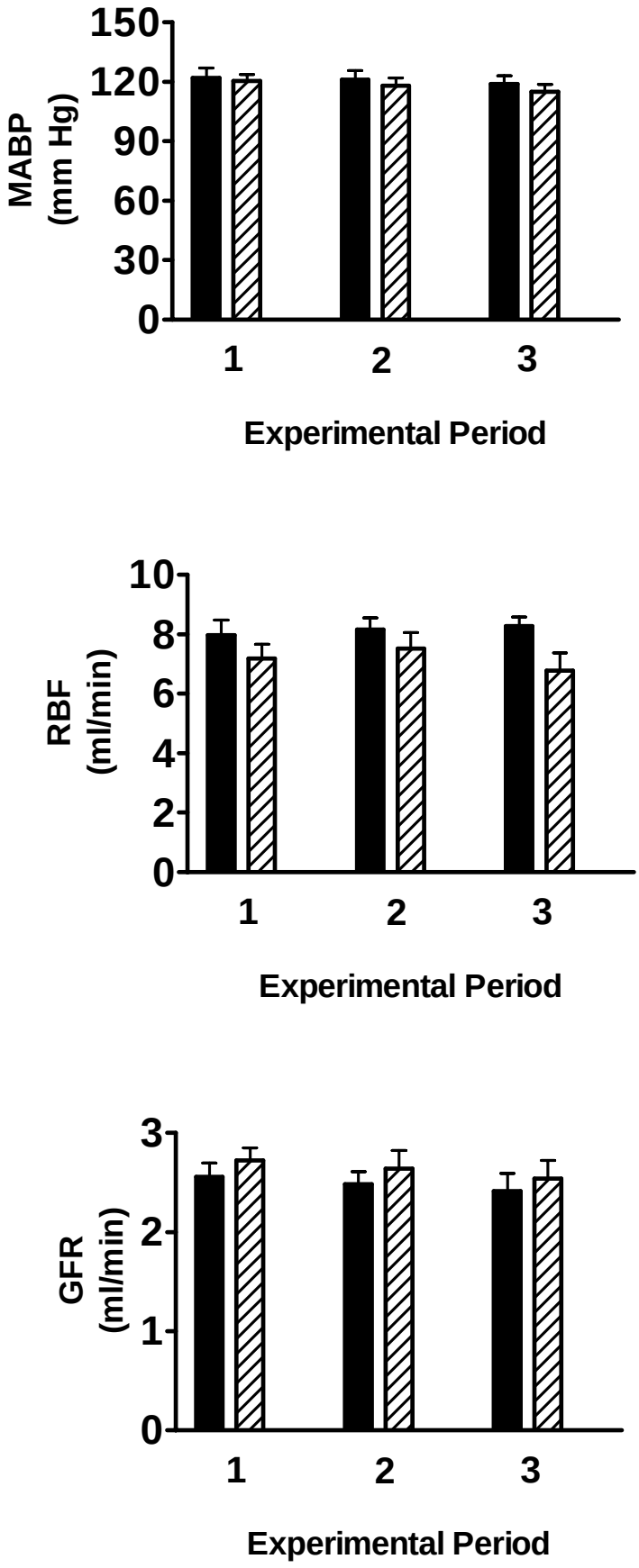

Figure 2

JPET Fast Forward. Published on October 6, 2006 as DOI: 10.1124/jpet.106.112748
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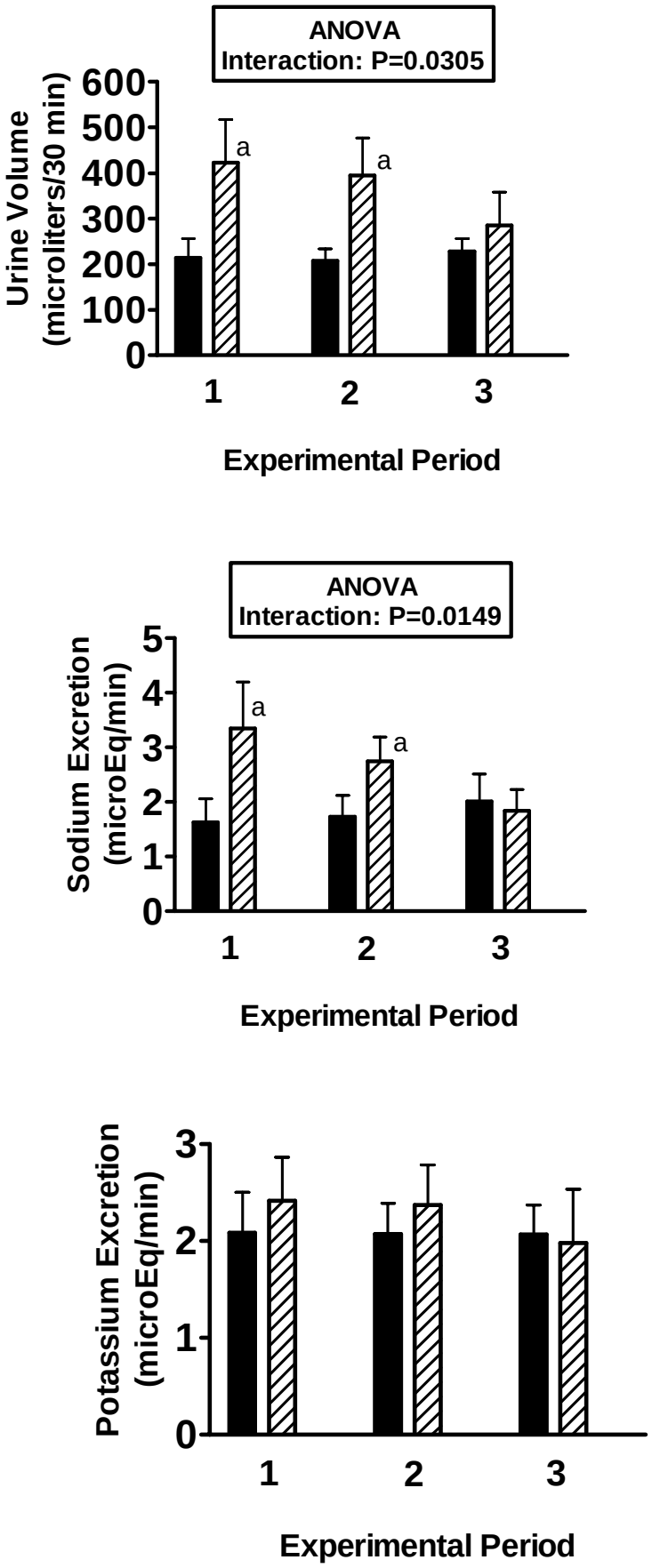

Figure 3

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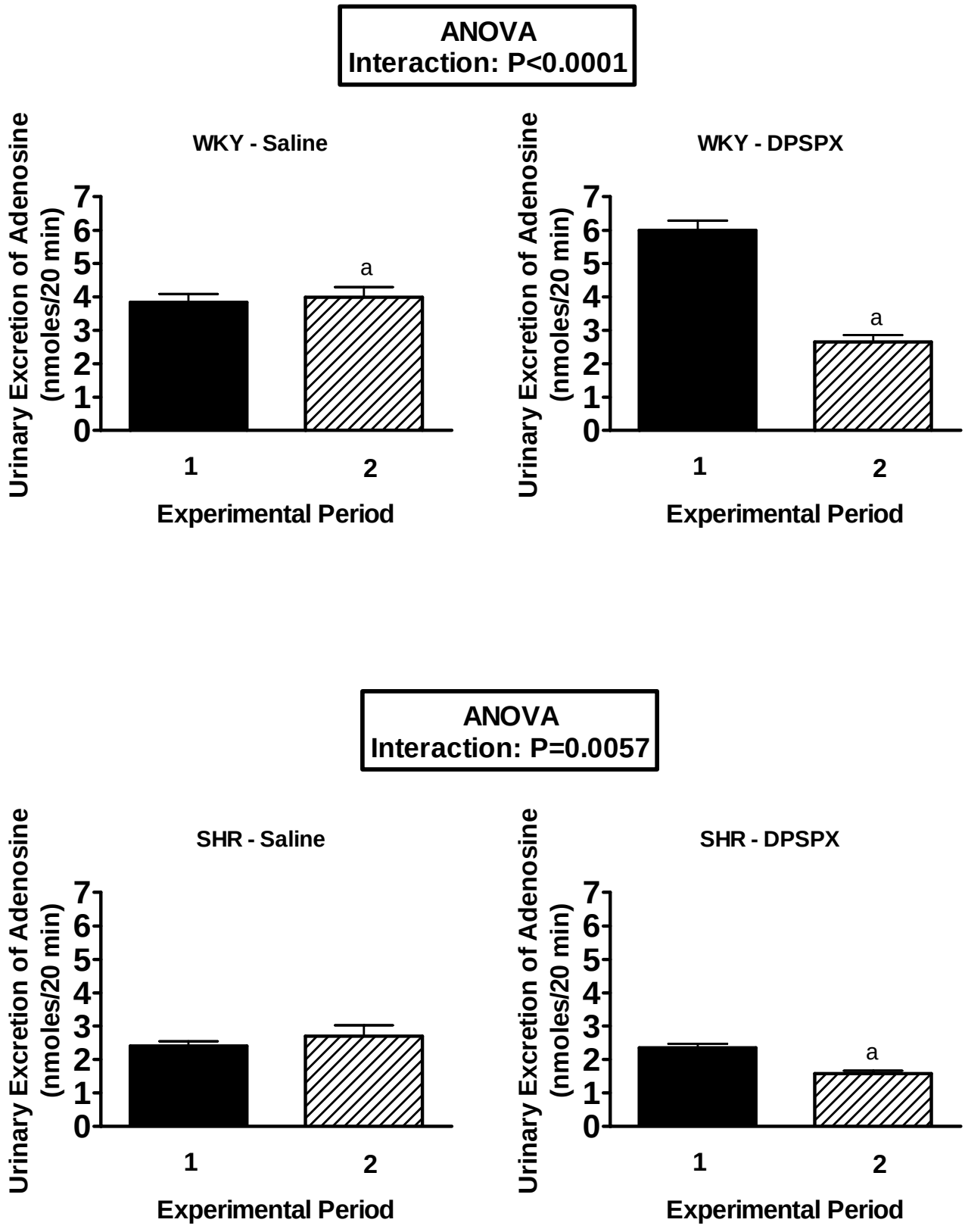

Figure 4

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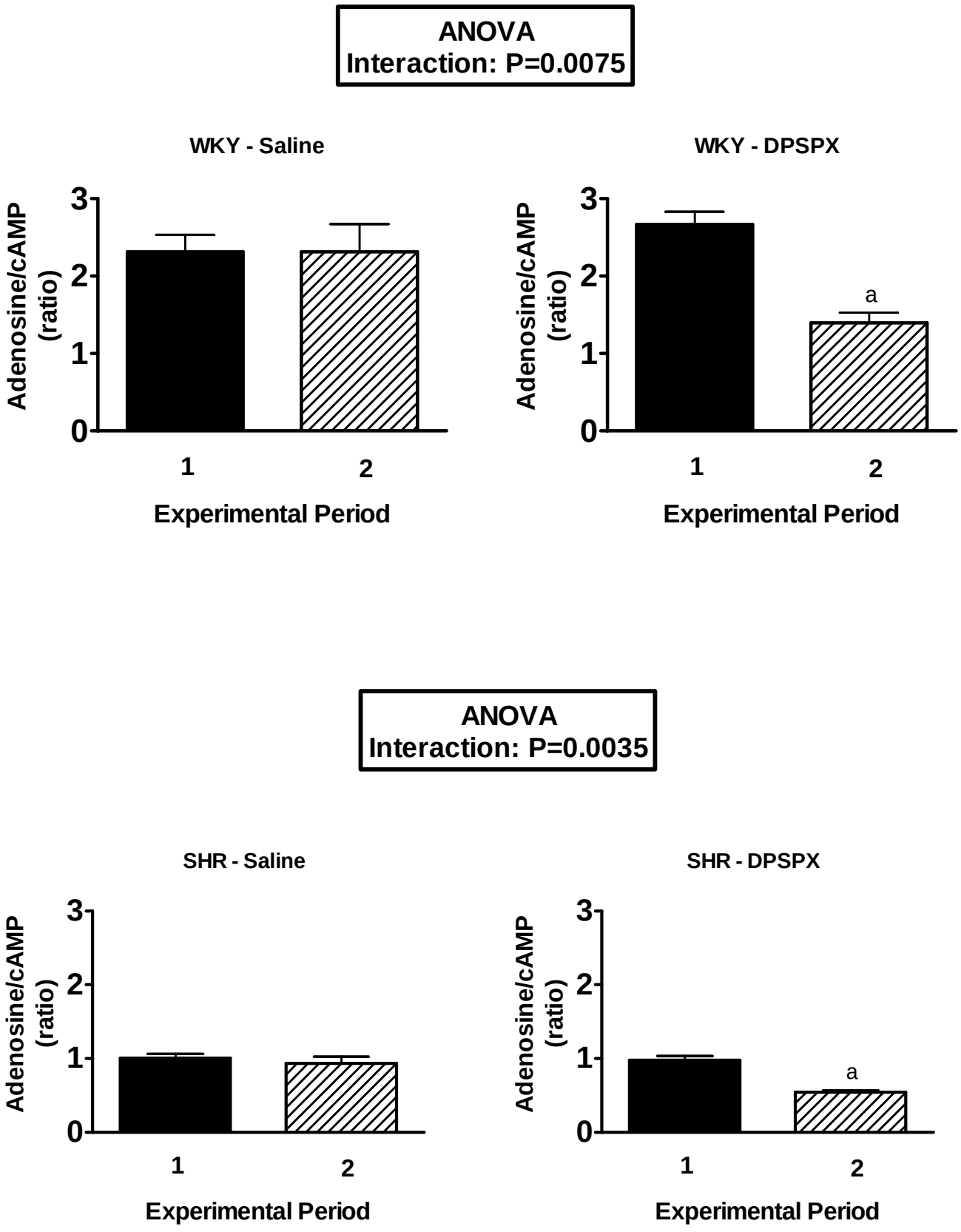

Figure 5