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A Study of the Effect of Isoproterenol on Red Blood Cell Concentrations of Adenine Nucleotides in a Freely Moving Rat Model *In Vivo**

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Abstract

Previous studies have shown that red blood cell (RBC) concentrations of adenine 5'-triphosphate (ATP) may be a key factor for post exercise effects responsible for cardiovascular protection. To test this concept further, we investigated the effect of isoproterenol on ATP metabolism in RBC using a freely moving rat model in vivo. Sprague Dawley rats were given either isoproterenol (30 mg/kg) or saline by subcutaneous (sc) injection. Blood samples were collected sequentially for up to 6 hours for measurement of adenine nucleotides in the RBC. Hemodynamic recordings were collected throughout the experiment. We have found isoproterenol induced 50% mortality under the experimental condition. It decreased systolic blood pressure (SBP) and diastolic blood pressure (DBP) immediately after the injection by -64 ± 22 and -64 ± 20 mmHg in less than 15 min, and increased HR steadily by +158 ± 59 bpm at the end of the experiment. Isoproterenol also increased RBC concentrations of adenine 5'-monophosphate (AMP) from 0.04 ± 0.01 to 0.28 ± 0.23 mM (+500%). The rats died had much greater breakdown of ATP to adenosine 5'-monophosphate (AMP) in the RBC than those surviving from the injury ($p<0.05$ for all the comparison).

Keywords: ATP; Biomarkers; Metabolism; Cardiovascular toxicity; Hemodynamic; Cardiovascular homeostasis; Rats

Introduction

Cardiovascular disease including stroke is the leading cause of death and disability worldwide [1-4] and an enormous economic burden to our societies [1,3,5-7]. Prevention by better diagnosis and drug treatment could provide a huge saving for the health care cost worldwide. Despite advancement in modern cardiovascular medicine, the prevalence of hypertension, ischemic heart disease (IHD) and stroke is still on the rise, and that finding an optimum therapy to slow disease progression remains a therapeutic challenge.

The importance of adenosine and adenosine 5'-triphosphate (ATP) in regulating many biological functions has long been recognized, especially for their effects on the cardiovascular system [8-17]. It is known that adenosine and ATP are key factors in regulation of coronary blood flow [12,18-20], inhibiting platelet aggregation [21], protection of myocardium [17,22-24], neuromodulation [25-32], attenuating tissue necrosis [14,33], ischemic preconditioning [34-39], immunomodulation [40], energy metabolism [16,41-43], and perhaps other functions as well (e.g. pain mediation) which maintain the homeostasis of the cardiovascular system. In respond to ischemia, ATP is broken down to release adenosine. The activity of adenosine is very short lived because it is rapidly taken up by myocardial and endothelial cells, red blood cells (RBC), and also rapidly metabolized to inosine and subsequently to hypoxanthine, adenine, S-adenosyl homocysteine (SAH), and other adenine nucleotides [8,18,44,45]. In our laboratory, we have been studying the potential of circulatory concentrations of adenosine and ATP, and their metabolites as biomarkers for cardiovascular protection and as targets for anti-ischemia drugs for several years [46-49]. It has been postulated that adenosine and ATP may be used as sensitive biomarkers to quantify myocardial and endothelial ischemia [8,44,50], and for monitoring therapeutic effects of anti-ischemia drugs [46,48,51-53]. More recently, we have shown that exercise improves cardiovascular hemodynamic and increases RBC concentrations of ATP and guanosine 5'-triphosphate (GTP) in a rodent model, particularly in the rats pre-treated with diltiazem (DTZ) [47,54,55], which was not observed in non-exercise rats [49]. The increase of circulatory concentrations of adenosine and ATP could be key factors for exercise preconditioning and a mechanism responsible for cardiovascular protection [17,34,56,57]. In order to study the importance of ATP metabolism in RBC in cardiovascular toxicity, we studied the effect of cardiovascular injury induced by isoproterenol on cardiovascular hemodynamic and RBC concentrations of adenine nucleotides in a freely moving rat model *in vivo* [58,59].

Materials and Methods

Chemicals

Authentic standards of Purine nucleotides including ATP, adenosine-5'-diphosphate (ADP), AMP, and isoproterenol hydrochloride were purchased from Sigma-Aldrich Chem Co. (St. Louis, MO, USA). Solvents were HPLC grade, and all other chemicals were reagent grade (Fisher Scientific, ON, Canada).

Animal study

The protocol followed the Canadian Council on Animal Care guidelines and was approved by the Dalhousie University Committee on Laboratory Animals. Sprague Dawley rats (SDR) with a carotid artery catheter Weighing between 250 and 320 g were used. They were acclimatized at the Carleton Animal Care Centre with free access to food and water for 48 hours before experiment. During experiment, each rat was kept in a freely moving caging environment with free access to drinking water (Figure 1). In the treatment group (n=10), after an hour settling in the cage, isoproterenol hydrochloride (30 mg/

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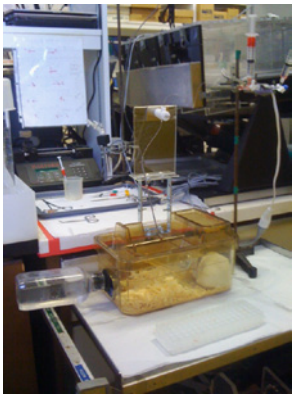

Figure 1: *In vivo* animal model.

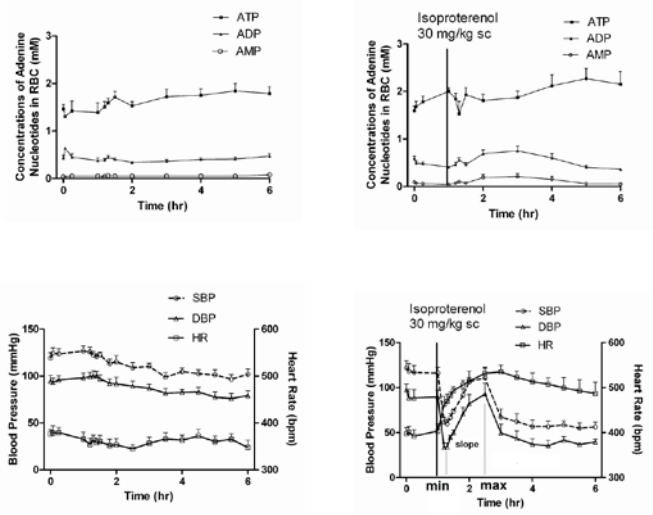

Figure 2: Cardiovascular effects of isoproterenol (30 mg/kg sc) *vs*. control (data are presented as mean ± SEM).

kg) freshly prepared in normal saline (30 mg/mL) was administered by subcutaneous (sc) injection in the dorsal area of the rat. A separate group receiving normal saline was used as control (n=9). Four blood samples (0.3 mL each) were collected from each rat via an indwelling catheter before isoproterenol (labeled as 0, 0.05, 0.25, and 1 hour), and then 7 more samples after isoproterenol labeled as 1.2, 1.5, 2, 3, 4, 5 and 6 hours. The blood samples were immediately mixed with a "Stopping Solution" for measurement of adenine nucleotides (ATP, ADP and AMP) [47]. Hemodynamic recordings including systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) were collected continuously throughout the experiment using a TruWave® disposable pressure transducer (Model PX601, Edwards Lifesciences Canada, Inc., Mississauga, ON, Canada) coupled to a Siemens hemodynamic monitor (Sirecust 400) and chart recorder (Siredoc) (Erlangen, FRG) [55]. The RBC samples collected were processed and lysed immediately using an ice cold 10% trichloroacetic acid. The lysate samples were stored at -80°C, and concentrations of ATP and other adenine nucleotides in the RBC were determined by a validated HPLC assay [47]. The rats which still survived at the end of the experiment (>5 hours after isoproterenol) were euthanized by cardiac puncture under general anesthesia with isoflurane.

Data analysis

Areas under the curve of RBC concentrations of ATP and other adenine nucleotides were calculated using trapezoidal method (Prism®-5, Graphpad Software Inc., La Jolla, USA). Maximum (Cmax) and minimum (Cmin) concentrations of adenine nucleotides and hemodynamic variables (SBPmax and min, DBPmax and min, HRmax and min, etc.) were obtained directly from the observed data (Figure 2). Hemodynamic and circulating biomarker variables between the control and isoproterenol treatment groups during the experiment were analyzed by student's paired and unpaired t-test, and differences considered significant when $p<0.05$. In addition, possible relationships between biomarkers from the group mean data and individual rat data were assessed using Pearson Correlation and linear regression analyses, and considered significant at $p<0.05$ (Minitab® Inc., Release 15.1, State College, PA, USA).

Results

Under the described experimental condition, isoproterenol induced 50% mortality within 5 hours after administration ($p<0.05$). It decreased SBP and DBP immediately after the injection (<15 min) by -64 ± 22 and -64 ± 20 mmHg (SBPmin and DBPmin), and increased HR by +158 ± 59 bpm at the end of the experiment ($p<0.05$). Both SBP and DBP rebounded to pre-treatment (baseline) level after 1-2 hours after injection (i.e. SBPmax and DBPmax) ($p<0.05$), and then fell again for the remaining of the experiment. There was no rebound from the HR response. In addition, isoproterenol also increased RBC concentrations of ADP and AMP immediately after injection, with corresponding decrease of ATP concentration (Figure 2). Two hours after the isoproterenol injection, RBC concentrations of AMP and ADP increased from 0.043 ± 0.0088 to 0.22 ± 0.19 mM (>400%) and 0.41 ± 0.067 to 0.75 ± 0.33 mM (>80%) ($p<0.05$ by paired t-test). The decrease of ATP concentration in the RBC immediately after isoproterenol was not statistically significant (1.97 ± 0.24 to 1.74 ± 0.45 mM; $p>0.05$ by paired t-test).

The rats that died (victims) had greater increase of the AMP and ADP concentrations than those surviving ones (survivors) after isoproterenol (Table 1). However, due to the small number of animal in the study (n=10), the difference found between the victims and survivors was not statistically significant. The same difference would have been

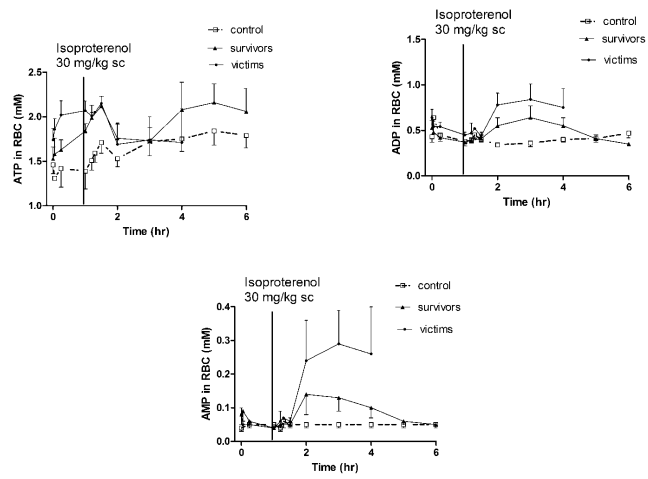

Figure 3: Effect of isoproterenol (30 mg/kg sc) on RBC concentrations of adenine nucleotides (data are presented as mean ± SEM).

significant if a larger sample size (e.g. n=20) was used. There was no significant difference in the RBC concentrations of ATP between the dying and surviving rats before or after isoproterenol (Figure 3), nor any difference in the hemodynamic responses to isoproterenol (SBP, DBP, and HR) between the victims and survivors (Figure 4). The suvivors from the insult appeared to have higher baseline blood pressure (SBP and DBP) and lower RBC concentrations of ATP before isoproterenol, but the differences were not statistically significant (Tables 1 and 2).

There were significant correlations between the mean RBC concentrations of ATP and ADP (r=-0.962, p<0.05) and between ATP and AMP (r=-0.957, p<0.05) after isoproterenol in the dying rats, but not in the surviving ones or in the control group (without isoproterenol) (Figure 5). There were also significant correlations between RBC

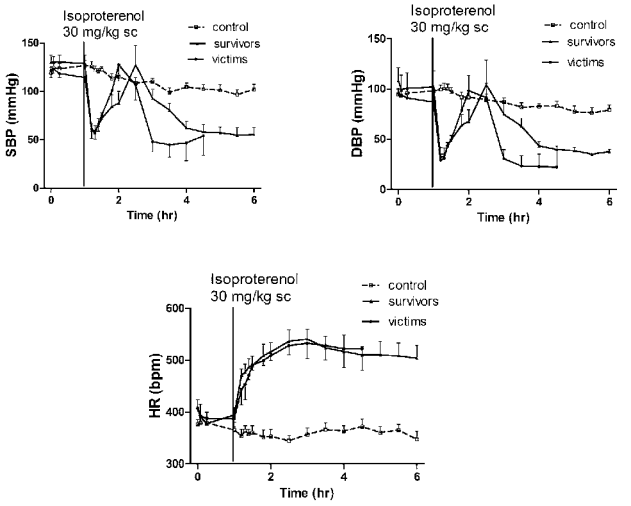

Figure 4: Effect of isoproterenol (30 mg/kg sc) on cardiovascular hemodynamic (data are presented as mean ± SEM).

Biomarker variables	Control (n=9)	Isoproterenol treatment (n=10)	Victims[a] (n=5)	Survivors[b] (n=5)
ATP $AUC_{(0\text{-}1\ hr)}$ (mM-hr)	1.56 ± 0.34[d]	1.84 ± 0.27	2.01 ± 0.25*	1.70 ± 0.18
ATP $AUC_{(1\text{-}4\ hr)}$ (mM-hr)	4.93 ± 0.97	5.19 ± 1.04	4.68 ± 0.99	5.55 ± 0.95
ATP Cmax(mM)[c]	2.12 ± 0.44	2.29 ± 0.37	2.13 ± 0.35	2.39 ± 0.36
ATP Cmin (mM)[c]	1.39 ± 0.27	1.46 ± 0.42	1.39 ± 0.52	1.61 ± 0.34
ADP $AUC_{(0\text{-}1\ hr)}$ (mM-hr)	0.42 ± 0.16	0.46 ± 0.10	0.51 ± 0.10	0.41 ± 0.08
ADP AUC(1-4 hr) (mM-hr)	1.11 ± 0.25	1.79 ± 0.64*	1.88 ± 0.84*	1.64 ± 0.49*
ADP Cmax(mM)	0.53 ± 0.12	0.81 ± 0.30*	0.94 ± 0.34*	0.65 ± 0.22
ADP Cmin (mM)	0.32 ± 0.08	0.48 ± 0.25	0.63 ± 0.30*	0.34 ± 0.05
AMP $AUC_{(0\text{-}1\ hr)}$ (mM-hr)	0.05 ± 0.03	0.05 ± 0.01	0.05 ± 0.02	0.06 ± 0.01
AMP $AUC_{(1\text{-}4\ hr)}$ (mM-hr)	0.14 ± 0.09	0.45 ± 0.39*	0.58 ± 0.52*	0.34 ± 0.21*
AMP Cmax (mM)	0.08 ± 0.04	0.28 ± 0.23*	0.38 ± 0.26*	0.16 ± 0.11
AMP Cmin (mM)	0.04 ± 0.03	0.10 ± 0.13	0.15 ± 0.17	0.04 ± 0.02

[a]Rats died within 5 hrs after isoproterenol
[b]Rats survived longer than 5 hrs after isoproterenol
[c]After isoproterenol or 1 hr for control
[d]Data represent mean ± SD
*p<0.05 *vs.* control (t-test)
**p<0.04 *vs.* victims (t-test)

Table 1: Comparison of RBC concentrations of adenine nucleotides in rats treated with isoproterenol (30 mg/kg sc) and control.

Hemodynamic variables	Control (n=9)	Isoproterenol treatment (n=10)	Victims[a] (n=5)	Survivors[b] (n=5)
SBP (mmHg)[c]	122 ± 12[e]	121 ± 15	114 ± 10	129 ± 18
SBPmin (mmHg)[d]	N/A	56 ± 16	57 ± 19	56 ± 14
SBPmax (mmHg)[d]	N/A	131 ± 34	133 ± 34	129 ± 38
SBPmax-SBPmin (mmHg)	N/A	75 ± 41	77 ± 46	73 ± 42
SBP-SBPmin (mmHg)	N/A	64 ± 22	58 ± 21	73 ± 23
Slope (mmHg/hr)	N/A	56 ± 29	61 ± 30	52 ± 31
DBP (mmHg)	94 ± 19	94 ± 22	87± 9	102 ± 32
DBPmin (mmHg)	N/A	30 ± 14	28 ± 12	31 ± 18
DBPmax (mmHg)	N/A	105 ± 36	105 ± 31	106 ± 46
DBPmax-DBPmin (mmHg)	N/A	76 ± 37	77 ± 38	75 ± 41
DBP-DBPmin (mmHg)	N/A	64 ± 20	59 ± 19	71 ± 22
Slope (mmHg/hr)	N/A	56 ± 30	57 ± 32	54 ± 33

[a]Rats died within 5 hrs after isoproterenol
[b]Rats survived longer than 5 hrs after isoproterenol
[c]Baseline or before isoproterenol
[d]After isoproterenol
[e]Data represent mean ± SD
*p<0.05 *vs.* control (t-test)
**p<0.04 *vs.* victims (t-test)

Table 2: Comparison of hemodynamic effect in rats treated with isoproterenol (30 mg/kg sc) and control.

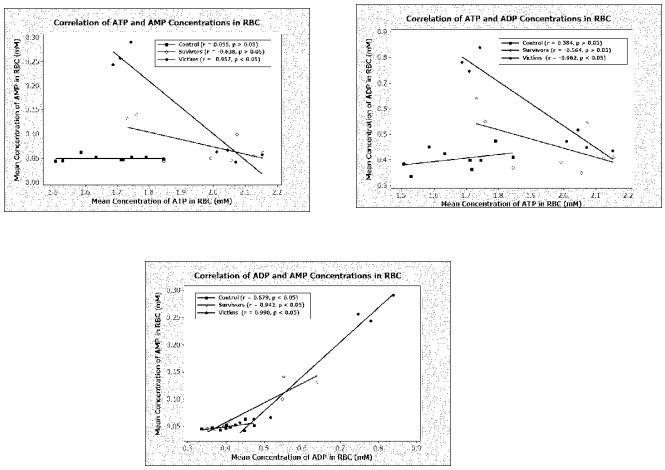

Figure 5: Correlations between mean RBC concentrations of adenine nucleotides.

concentrations of ADP and AMP in the rats treated with isoproterenol and in the control group (Figure 5). When individual rat data were analyzed, the regression coefficient (β) and correlation coefficient (r) between the adenine nucleotides were significantly different between the isoproterenol treated rats and the controls. Further, the rats died from the insult had significantly greater breakdown of ATP to AMP than those survived (β=-0.318 ± 0.190 *vs.* -0.058 ± 0.115, p<0.05) (Table 3).

Discussion

Isoproterenol is known for many years as a stress agent of the sympathetic system which increases HR, but the effects on BP particularly DBP are highly variable [58,60]. After 50–200 mg/kg dose given once daily by subcutaneous (sc) injection for 2 days to rats, isoproterenol has been shown to induce cardiac hypertrophy, increase serum concentrations of lactate dehydrogenase (LDH), creatine kinase (CK), aspartate aminotransferase (AST), serum cardiac troponin I and

Biomarker variables	Control (n=9)	Isoproterenol treatment (n=10)	Victims[a] (n=5)	Survivors[b] (n=5)
ATP *vs*. AMP r[c]	-0.051 ± 0.312*,[e]	-0.515 ± 0.421	-0.717 ± 0.362	-0.262 ± 0.380
ATP *vs*. AMPβ[d]	0.002 ± 0.032*	-0.202 ± 0.204	-0.318 ± 0.190	-0.058 ± 0.115**
ATP *vs*. ADP r	0.299 ± 0.306*	-0.277 ± 0.569	-0.429 ± 0.654	-0.088 ± 0.456
ATP *vs*. ADP β	0.103 ± 0.117*	-0.294 ± 0.542	-0.523 ± 0.642	-0.008 ± 0.194
ADP *vs*. AMP r	0.579 ± 0.260	0.787 ± 0.253	0.812 ± 0.248	0.767 ± 0.285
ADP *vs*. AMP β	0.132 ± 0.124*	0.392 ± 0.277	0.296 ± 0.282	0.469 ± 0.278

[a]Rats died within 5 hrs after isoproterenol
[b]Rats survived longer than 5 hrs after isoproterenol
[c]Correlation coefficient
[d]Regression coefficient
[e]Data represent mean ± SD
*p<0.05 *vs*. isoproterenol (t-test)
**p<0.04 *vs*. victims (t-test)

Table 3: Correlation between RBC concentrations of adenine nucleotides in rats after isoproterenol (30 mg/kg sc).

a host of lipids and triglycerides [58,59,61,62]. Lower dose (5 mg/kg) injected daily for a week has been shown to increase different cardiac fibrotic and hypertrophic markers, and induce cytochrome P-450 enzymes [63]. The ability to titrate dosage of isoproterenol and induce cardiovascular injury quickly without surgical manipulation makes it particularly suitable as a working animal model for study of acute cardiovascular toxicity [59,64,65].

Under the experimental conditions, 50% of the rats died within 5 hours after isoproterenol, while there was no mortality in the control group. It should be pointed out that as much as 11 blood samples (close to 3.5 ml) were obtained from each rat in both control and isoproterenol treatment groups, which could add to the significant mortality rate observed in the treatment group. We have shown in this study that immediately following a single 30 mg/kg subcutaneous injection of isoproterenol, both SBP and DBP fell by 50% or more, and HR by more than 100 bpm. There was a rebound of the blood pressures to close to pre-treatment level 1-2 hour after the isoproterenol injection, following that they both fell again and remained much lower than the pre-treatment level. However, there was no rebound of the HR which remained substantially elevated by over 150 bpm at the end of the experiment (Figure 2). It is important to note that all the deaths occurred after the rebound and those survived from the insult also had significantly lower blood pressure and elevated HR at the end of the experiment similar to the victims (Figure 4). There was no significant difference in hemodynamic response between the survivors and victims (Table 2).

In addition to the hemodynamic effects, there was also an immediate increase of RBC concentrations of ADP and AMP, with a corresponding decrease of ATP concentrations after the isoproterenol injection (Figure 2). While the increase of ADP and AMP concentrations were significant ($p<0.05$), the decrease of ATP concentrations was not. This was probably attributed to the much higher concentrations of ATP in the RBC (5-10 times) such that a relatively small decrease of ATP concentration can lead to much greater increase of ADP and AMP concentrations in the RBC. The results suggested that ATP was broken down to ADP and AMP in the RBC after isoproterenol, which is known to occur in ischemia [8,18,44]. The fact that it happened in the RBC *in vivo* after isoproterenol injection suggests a unique role of ATP metabolism in RBC for cardiovascular homeostasis. It has been proposed that RBC may serve as oxygen sensor in the cardiovascular system [66,67]. It is known that RBC is capable of releasing increased amounts of ATP as oxygen content falls and its haemoglobin becomes desaturated [68]. Thus it is conceivable that RBC may sense tissue oxygen requirements when they travel through the microcirculation and release vasodilatory compounds such as ATP and its metabolites that enhance blood flow in hypoxic tissues [67]. The released adenine nucleotides would help to increase blood supply to the tissue and preserve an optimum balance between oxygen supply and demand, thereby modulating the concentrations of tissue ATP within the cardiovascular system. Such a mechanism would eliminate the requirement for a diverse network of sensing sites throughout the vasculature, and should provide a more efficient means of appropriately matching oxygen supply with demand. It is important to note that while there was no difference in the hemodynamic response between the victims and survivors, the RBC concentrations of AMP was considerably higher in the dying rats (Figure 3). However, due to the small number of rats (n=10) used in the pilot experiment, the difference was below significant level ($p=0.12$). Increasing the number of rats to n=20 could result in significant difference ($p=0.024$).

In order to identify more sensitive biomarkers predictive of mortality induced by isoproterenol, individual and group mean data were analyzed by association analyses. There were significant correlations between the mean RBC concentrations of ATP and ADP ($r=-0.962$, $p<0.05$); and also between ATP and AMP ($r=-0.957$, $p<0.05$) in the dying rats, but not in the surviving ones (Figure 5), suggesting that more ATP was broken down to ADP and AMP in the dying rats. Analysis of individual rat data supported this thesis although only the regression coefficient (β) between ATP and AMP showed significant difference between the victims and survivors (Table 3). The current study suggests that ATP metabolism in the RBC may be sensitive biomarkers predictive of cardiovascular mortality. We have recently shown that a brief exercise (15 minutes on a treadmill at a speed of 10 m/min) induced a greater post-exercise hypotension in spontaneously hypertensive rats (SHR) compared to SDR. It also decreased ATP concentrations in the RBC during exercise in the SHR as opposed to an increase found in SDR. This could be attributed to a reduced energy reserves in the SHR which could imply SHR may be more vulnerable to ischemia injury [69]. The finding is very encouraging as most of the current cardiac biomarkers are physiological and/or pathological markers specific to cardiac tissues and have limited predictive values for cardiovascular mortality [70-72]. However, further study using larger sample size in SDR and SHR to confirm the role of ATP metabolism in RBC for cardiovascular homeostasis is warranted. Significant correlations between RBC concentrations of ADP and AMP were also observed (Figure 5). However, the relationship was not predictive of mortality and not specific for cardiovascular toxicity (Table 3). In summary, we have shown for the first time isoproterenol acutely induced breakdown of ATP to ADP and AMP in RBC *in vivo,* which may be the rate limiting step for cardiovascular toxicity.

Conclusion

Isoproterenol profoundly altered cardiovascular hemodynamic and induced break down of ATP in the RBC to ADP and AMP particularly in the dying rats. Association between RBC concentrations of ATP with AMP or ADP may be used as sensitive and predictive biomarker for cardiovascular mortality.

Acknowledgement

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