Lack of Reactive Oxygen Species Deteriorates Blood Pressure Regulation in Acute Stress

I. BERNAŤOVÁ¹, P. BALIŠ¹, R. GOGA², M. BEHULIAK³, J. ZICHA³, I. SEKAJ²

¹Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Bratislava, Slovak Republic, ²Institute of Robotics and Cybernetics, Faculty of Electrical Engineering and Information Technology, Slovak University of Technology, Bratislava, Slovak Republic, ³Institute of Physiology of the Czech Academy of Sciences, Prague, Czech Republic

Received May 14, 2016
Accepted September 5, 2016

Summary
This study investigated the contribution of reactive oxygen species (ROS) to blood pressure regulation in conscious adult male Wistar rats exposed to acute stress. Role of ROS was investigated in rats with temporally impaired principal blood pressure regulation systems using ganglionic blocker pentolinium (P, 5 mg/kg), angiotensin converting enzyme inhibitor captopril (C, 10 mg/kg), nitric oxide synthase inhibitor L-NAME (L, 30 mg/kg) and superoxide dismutase mimeticum tempol (T, 25 mg/kg). Mean arterial pressure (MAP) was measured by the carotid artery catheter and inhibitors were administered intravenously. MAP was disturbed by a 3-s air jet, which increased MAP by 35.2±3.0 % vs. basal MAP after the first exposure. Air jet increased MAP in captopril- and tempol-treated rats similarly as observed in saline-treated rats. In pentolinium-treated rats stress significantly decreased MAP vs. pre-stress value. In L-NAME-treated rats stress failed to affect MAP significantly. Treatment of rats with P+L+C resulted in stress-induced MAP decrease by 17.3±1.3 % vs. pre-stress value and settling time (20.1±4.2 s). In P+L+C+T-treated rats stress led to maximal MAP decrease by 26.4±2.2 % (p<0.005 vs. P+L+C) and prolongation of settling time to 32.6±3.3 s (p<0.05 vs. P+L+C). Area under the MAP curve was significantly smaller in P+L+C-treated rats compared to P+L+C+T-treated ones (167±43 vs. 433±69 a.u., p<0.008). In conclusion, in rats with temporally impaired blood pressure regulation, the lack of ROS resulted in greater stress-induced MAP alterations and prolongation of time required to reach new post-stress steady state.

Key words
Air-jet stress • Superoxide • Nitric oxide • Sympathetic nervous system • Renin-angiotensin system

Corresponding author
I. Bernatova, Institute of Normal and Pathological Physiology, Slovak Academy of Sciences, Slenkiewiczova 1, 813 71 Bratislava, Slovak Republic. E-mail: iveta.bernatova@savba.sk

Introduction

Blood pressure (BP) is one of the principal vital signs and the maintenance of normal BP is critical in order to keep optimal oxygenation of tissues and organs in all vertebrates, including humans. That is why BP is continually controlled and regulated on optimal levels by the central, peripheral and local tissue mechanisms. All these levels of BP regulation, including endothelium-derived factors, play a significant role in the modulation of final BP level. However, among all components of BP regulatory system the sympathetic nervous system (SNS), renin-angiotensin system (RAS) and nitric oxide (NO) seem to be the most powerful regulators.

When exposed to sudden stressor the autonomic nervous system is activated for fight or flight reaction. The stress-induced increase in heart rate (HR) by sympathetic stimulation of cardiac β-adrenergic receptors is accompanied by a rise in BP mediated mainly by α-adrenergic stimulation. On the other hand, NO is the major depressor factor attenuating BP. In fact, NO, well
known neurotransmitter, neuromodulator and vasodilator, has to be released continually, to act against vasoconstriction produced by SNS and RAS, and relative NO-deficiency was observed in experimental hypertension (Kunes et al. 2004). Furthermore, all noradrenaline, NO and angiotensin II, play a significant role in BP regulation at both central and peripheral levels (Fisher et al. 2009, Reid 1992, Pechanova 2010) affecting also stress-induced pressor responses (Cordellini et al. 2006, Porter 2000). Thus, in order to maintain optimal BP, there is a significant cross-talk among these three systems, at all levels of BP regulation, under normal conditions as well as during stress.

Reactive oxygen species (ROS), mainly superoxide, were shown to interact with RAS, SNS and NO. ROS can be produced by mitochondria, uncoupled NOS and by various oxidases (Majzunova et al. 2013). ROS were shown to activate SNS (Campese et al. 2004), the activation of RAS was shown to stimulate ROS production (Touyz 2004, Tsuda 2012) and superoxide deactivates NO in direct chemical reaction (Thomson et al. 1995), which limits NO bioavailability. Elevated levels of ROS were shown in various experimental models of hypertension as well as in hypertensive humans as described below. Unfortunately, although chronic treatment with antioxidants reduced BP in various experimental models of hypertension (Bernatova et al. 2002, Galleano et al. 2013, Vaneckova et al. 2013, Vasdev et al. 2002a,b) long-term antioxidant treatment in clinical trials produced variable cardiovascular effects (Kris-Etherton et al. 2004). Thus, the contribution of ROS-mediated mechanisms to BP regulation, both acute and chronic, is still unclear. In addition, it is very difficult to determine the exact ROS-mediated contribution to BP regulation in the presence of other major BP regulatory systems because the effect of ROS seems to be rather mild.

The aim of this study was to determine the contribution of superoxide to BP regulation in conscious normotensive rats, in which BP was disturbed by acute air-jet stress. To emphasize the role of superoxide, the function of other regulatory systems (SNS, RAS and NO) was pharmacologically attenuated. Then the integrated pressor/depressor responses induced by air-jet in freely moving rats were recorded and maximal mean arterial pressure (MAP) response, settling time and area under the BP curve were determined in order to characterize the ability of organism to maintain BP at new post-stress level.

**Methods**

All procedures and experimental protocols were approved by the Ethical Committee of the Institute of Physiology CAS and conformed to the European Convention on Animal Protection and Guidelines on Research Animal. Adult 12-16-week male Wistar rats were used in this study. BP was determined using the catheter inserted into the carotid artery. One day before experiment, polyethylene catheters were inserted into the left carotid artery (PE 50) and the jugular vein (PE 10) under 2 % isoflurane anesthesia and exteriorized in the interscapular region as described previously (Behuliak et al. 2011). Rats were allowed to recover from anesthesia for approximately 20-24 h. All experiments were performed in quiet room to avoid any non-specific stimuli affecting BP. Before experiments the rats were placed into dark plastic box of 19 x 11 x 7 cm size, attached to BP recording device and BP was allowed to stabilize for at least 30 min. The BP was recorded in conscious rats using the PowerLab system (ADInstruments, Bella Vista, Australia). Systolic BP (SBP) and diastolic BP (DBP) were recorded with sampling rate 400 samples/s (i.e. sampling frequency 400 Hz). Mean arterial pressure (MAP) was calculated as MAP = DBP + 1/3(SBP - DPB). MAP data were transformed into Matlab® environment for further analysis.

Acute stress was produced by a 3-s pulse of air at the face of rat as described previously with a minor modification of exposure time (Nakamoto et al. 2007). To attenuate particular regulation systems, the following drugs were used: SNS was inhibited by a ganglionic transmission blocking agent pentolinium (P) at a dose of 5 mg/kg. RAS was attenuated by the inhibition of the angiotensin-converting enzyme by captopril (C) at a dose of 10 mg/kg. NO production was attenuated by the inhibition of nitric oxide synthase using NG-nitro-L-arginine methyl ester (L-NAME, L) in a dose of 30 mg/kg. The superoxide level was reduced by tempol (T), a radical scavenger that mimics the activity of superoxide dismutase, at a dose of 25 mg/kg. All substances were dissolved in saline (S) and given to circulation via the catheter inserted into the jugular vein as i.v. bolus in a volume 1 ml/kg of body weight as described previously (Behuliak et al. 2011). All chemicals were from Sigma (Germany).
Protocols of the experiment (Fig. 1)

Protocol 1. Effect of stress repetition: Possible attenuation of the air-jet-induced changes of MAP was investigated by three repetitions of air jet in 10-min intervals in 10 rats (Figs 2A, B, C).

Protocol 2. The effect of individual inhibitors (i.e. drugs) was investigated as follows: After 30 min stabilization of BP, rats were exposed to air jet to determine the response of each rat to this stimulus. Then BP was allowed to stabilize for 10 min. The effect of individual inhibitors was detected by administration of S, P, L, C and T, respectively. Basal MAP was determined in stable 10-s period, 1 min before drug administration, and then during the tenth min after drug administration. Then rats were exposed to the second air jet to determine the effect of the individual substances on air-jet-induced MAP responses. MAP was determined between 1-5 s after stress as the maximal change of BP (Fig. 3).

Protocol 3. At the end of the protocol 2, the next two inhibitors (different from the first one and T) were administered in random order in 10-min intervals. After the third inhibitor, BP was allowed to stabilize for next 10 min. Average 10-s value of MAP determined during the tenth min after the third inhibitor (i.e. after the administration of P+C+L) was taken as pre-stress MAP value. Then the third 3-s air jet was delivered and MAP recovery transient process was continuously recorded for 10 min (Fig. 4A).

Protocol 4. In a separate group of rats the protocol 2 and 3 were repeated but T was administered as the fourth substance (i.e. P+C+L+T) before the third air jet (Fig. 4B).

Air-jet stress-induced MAP time-responses were expressed as ΔMAP (%) with respect to MAP determined during the approximately 20-s period before the respective air-jet stress, i.e. the average MAP before stress was calculated from approximately 8000 samples. The responses were analyzed by determination of the following three parameters: 1) the maximal stress-induced peak deviation of MAP (P_{max}), 2) settling time (t_{s}) expressed in seconds, 3) area under the MAP curve (AUC), expressed in arbitrary units (a.u.) and 4) maximal MAP deviation detected during the post-stress steady state (δ), expressed as percentage change with regard to P_{max} (Fig. 2A).

P_{max} was defined as the difference between the maximal stress-induced MAP compared to MAP determined during the 20-s period before the given air-jet stress. Settling time (t_{s}) was defined as the time interval between the start of the air-jet stress and the moment when the MAP reached and stayed within the MAP error band E_{ss} (Tay et al. 1997). E_{ss} was defined as the interval (MAP_{ss} - δ; MAP_{ss} + δ) where δ represents the maximal MAP deviation from the average MAP during the steady state (MAP_{ss}). In this study, MAP_{ss} was determined during the time period between approximately 40-60 s.
after air jet, and it was calculated as the average MAP value from approximately 8000 samples of MAP. Because the oscillation amplitudes of MAP during the steady state were variable in particular rats, the size of $\delta$ was determined in each rat individually.

The area under the MAP curve (AUC) was calculated as the area which is bounded by the MAP curve and by the MAP<sub>ss</sub> value during the settling time $t_s$, expressed in arbitrary units

$$AUC = \int_0^{t_s} (|MAP - MAP_{ss}|)$$  \hspace{1cm} (1)

The equation (1) describes the continuous-time measurement evaluation. In this study the MAP was measured in discrete form with the sampling period $T$, where $T=1/f$ and the sampling frequency $T = 400$ Hz. In the discrete-time domain the equation (1) can be transformed to

$$AUC = \sum_{i=1}^{N} T(|MAP_i - MAP_{ss}|)$$ \hspace{1cm} (2)

where $N$ is the number of samples during settling time $t_s$.

**Statistical analysis**

The effect of repeated air-jet exposure and the effect of individual inhibitors and stress were analyzed by one-way ANOVA and Dunnet’s post-hoc test. Between the groups differences (L+P+C vs. L+P+C+T) were analyzed using Student’s t-test for independent samples. The significance level was set at 5% ($\alpha=0.05$). All values were presented as the mean ± SEM. Data were analyzed with Statistica® 7.0 (Stat Soft, Inc., USA), GraphPad Prism 5.0 (GraphPad Software, Inc., USA) and Matlab® R2010a (Mathworks, Inc, USA).

**Figure 2.** Average waveforms (black line) of the air-jet stress-induced changes of mean arterial pressure (MAP) and its standard error of mean (SEM, thin line) after the first (A), the second (B) and the third (C) air-jet stress, n=10. AUC – area under the blood pressure curve, $E_{\delta}$ – MAP<sub>ss</sub> error band, $MAP_{ss}$ – MAP steady state, $P_{max}$ – maximal $\Delta$MAP vs. pre-stress, $t_s$ – settling time. See graphic explanations of the $P_{max}$, $t_s$ and AUC in the Figure 2A.

**Table 1.** Characteristics of mean arterial pressure regulation induced by air-jet stress itself (Stress 1 – Stress 3) and air-jet stress in the P+L+C- and P+L+C+T-treated rats.

<table>
<thead>
<tr>
<th>Group</th>
<th>$P_{max}$ (%)</th>
<th>$t_s$ (s)</th>
<th>AUC (a.u.)</th>
<th>$\delta$ (%) $P_{max}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stress 1 (n=10)</td>
<td>35.2±3.0</td>
<td>8.2±1.2</td>
<td>143±31</td>
<td>17.3±2.2</td>
</tr>
<tr>
<td>Stress 2 (n=10)</td>
<td>28.4±3.4</td>
<td>8.4±2.1</td>
<td>128±33</td>
<td>20.1±3.1</td>
</tr>
<tr>
<td>Stress 3 (n=10)</td>
<td>23.3±3.0</td>
<td>9.4±1.9</td>
<td>93±17</td>
<td>28.7±2.8*</td>
</tr>
<tr>
<td>P + L + C (n=8)</td>
<td>-17.3±1.3</td>
<td>21±4</td>
<td>167±43</td>
<td>-6.6±0.9</td>
</tr>
<tr>
<td>P + L + C + T (n=10)</td>
<td>-26.4±2.2*</td>
<td>33±3*</td>
<td>433±69+</td>
<td>-10.3±2.8*</td>
</tr>
</tbody>
</table>

$P_{max}$ – maximal mean arterial pressure (MAP) change in percentage of pre-stress MAP, $t_s$ – settling time, AUC – area under the blood pressure curve, $\delta$ – maximal MAP deviation from the average value of post-stress MAP, L – N<sup>-</sup>-nitro-L-arginine methyl ester, P – pentolinium, C – captopril, T – tempol. Results are mean ± SEM. * $p<0.05$ vs. Stress 1, * $p<0.05$ vs. the P+L+C group.
Results

The first air-jet-induced MAP responses were relatively consistent in saline-treated control rats with $P_{\text{max}}$ about 35% vs. pre-stress value, settling time about 8 s, AUC approximately 143 a.u. and steady-state error $\delta$ about 17% of $P_{\text{max}}$ (Fig. 2A, Table 1). These parameters did not change significantly after the second air-jet stress (Fig. 2B, Table 1). The third exposure to air jet led to the significant reduction of the $P_{\text{max}}$ ($p<0.04$) and elevation the steady-state error ($p<0.04$) vs. the first stress, while the other two parameters were altered non-significantly (Fig. 2C, Table 1).

Saline itself had no effect on basal MAP and stress-induced MAP changes compared to non-injected rats (Fig. 3). Ten minutes after the administration of pentolinium or captopril, MAP decreased by approximately 25% and 10% vs. basal values, respectively, ($p<0.05$ for both). L-NAME itself increased MAP by approximately 38% vs. basal values ($p<0.001$). Tempol itself failed to affect MAP significantly (Fig. 3).

Exposure of captopril-treated rats to air jet led to approximately 16% increase of MAP vs. pre-stress levels ($p<0.05$) (Fig. 3). Similarly, MAP of tempol-treated rats exposed to air jet increased by approximately 29% vs. pre-stress levels ($p<0.01$). In pentolinium-pretreated rats MAP decreased significantly by approximately 17% vs. pre-stress levels ($p<0.01$). Interestingly, in L-NAME-treated rats the acute stress failed to affect MAP significantly, however, a decreasing trend of MAP was observed in these rats (Fig. 3).

Pretreatment of rats with P+L+C led to stress-induced MAP response characterized by maximal decrease of MAP by about 17% vs. pre-stress, $t_s$ approximately 21 s and AUC about 167 a.u. (Fig. 4A, Table 1). Co-administration of tempol to P+L+C-treated rats let to more pronounce stress-induced changes of MAP vs. the P+L+C group in all parameters determined (Fig. 4B, Table 1).
Discussion

This study investigated the contribution of ROS to regulation of BP in stress reaction induced by sudden aversive stimulus produced by air jet. We found that in the rats in which major BP regulatory systems were ineffective, the lack of ROS resulted in greater changes of MAP and prolongation of settling time compared with those determined in rats with unaltered ROS level. As this observation was done after acute air-jet stress in rats in which SNS, RAS and NO-mediated mechanisms were attenuated, it is assumed that the effect of ROS could be associated with other regulatory mechanisms related to the local endothelial factors.

We used acute air jet as the stimulus to induce stress reaction associated with prominent BP increase. Air-jet stress is an environmental stressor resulting in psychoemotional stress, which causes the characteristic pattern of the classic defense reaction with an increase in MAP, heart rate and renal sympathetic nerve activity (Davisson et al. 1996, Kanbar et al. 2007, Yamazato et al. 2006).

In this study, the second repetition of air jet was associated with only non-significant reduction of maximal MAP increase, settling time and area under the MAP curve, i.e., parameters that characterize the return of BP to new post-stress equilibrium. However, the third air-jet stress led to reduction of maximal MAP increase suggesting gradual habituation of rats to this stimulus. On the other hand, no reductions of BP responses were observed using 1-s air jet in 5-min interval (Davisson et al. 1996) or when the stimulus was delivered in different days (Fuchs et al. 1998). Significant habituation, which is a fundamental learning in which the organism learns not to respond to redundant non-significant stimuli (Groves and Thompson 1970), was observed in all parameters determined after the fifth exposure to air jet in our preliminary study (data not shown).

There is evidence that interaction of RAS and ROS play an important role in the control of cardiovascular functions, including stress-induced cardiovascular responses. In this study, captopril reduced baseline MAP significantly. On the other hand, tempol had only minor effect on baseline BP. In rats pretreated with intravenous administration of captopril and tempol, respectively, air jet led to BP increase similar to that observed in saline-treated rats, suggesting that RAS and ROS in normotensive rats had no critical impact on stress-induced pressor responses in this experimental setup. In previous studies, tempol administered intravenously caused dose-dependent reductions in blood pressure of hypertensive rodent models but less effect was found in normotensive rats (Walcox and Pearlman 2008, Zicha et al. 2001). In rabbits, tempol-pretreatment reduced angiotensin II-induced pressor responses as well as air-jet-induced MAP increase (Mayorov et al. 2004).

Inhibition of ganglionic transmission by pentolinium, which inhibits nicotinic acetylcholine receptors in sympathetic ganglia and thus inhibits the release of noradrenaline from nerve terminus, was associated with decrease of basal BP and inversion of air-jet-induced MAP response. After the blockade of nicotinic receptors by pentolinium the smooth muscle relaxation and BP decrease may occur due to β2-receptor-mediated vasorelaxation likewise it was observed in cocaine-induced BP responses (Poon and van den Buuse 1998). Thus SNS has clearly decisive influence on air-jet stress-induced MAP responses. Similar findings on the role of SNS in air-jet stress-induced MAP changes were observed using losartan (angiotensin II receptor antagonist) and prazosin (α1-adrenoceptor blocker) (Gaudet et al. 1996).

Interestingly, air-jet stress failed to increase MAP when rats were pre-treated with L-NAME. As a lack of NO due to chronic NOS inhibition is associated with elevation of BP (Bernatova et al. 2002), one would expect rather more pronounced increase of BP in L-NAME-treated vs. saline-treated rats than a mild BP decrease detected in our study. The absence of MAP increase after air jet was observed also by Davisson et al. (1996) who found that L-NAME did not interfere with the central processing of the air-jet or the resultant changes in autonomic nerve activity. The authors suggested the release of pre-existing or de novo synthesized NO-related factor(s) from vascular endothelium and postganglionic NO synthase-containing sympathetic nerves, which may regulate vascular tone under conditions in which NO synthesis is temporarily compromised (Davisson et al. 1996). The absence of air-jet-induced BP increase after L-NAME-treatment was observed also by Moraes-Neto et al. (2014) who observed attenuated BP rise following a 60-min restraint stress after bilateral injection of an NMDA receptor antagonist, NOS inhibitor or NO scavenger into the dorsal hippocampus. Their results suggest that the activation glutamatergic and nitrergic neurotransmission within the hippocampus may play a role in autonomic modulation acute stress (Moraes-Neto et al. 2014). Thus the exact...
mechanism by which L-NAME attenuates pressor responses induced by acute stress needs further investigations.

This study was focused on determination of the role of ROS in BP regulation in terms of maximal stress-induced BP changes and time required to achieve a new balance as well as by δ coefficient that represents the maximal MAP deviation from the average MAP during the post-stress steady state. These parameters are usually used to characterize regulation processes, i.e. ability of the system to keep given variable in defined range of values. By now ROS were investigated and discussed mainly as one of the factors involved in development and/or maintenance of high blood pressure as increased levels of ROS may lead to oxidative stress. Oxidative stress was observed in patients with essential hypertension, renovascular hypertension, malignant hypertension or pre-eclampsia (Higashi et al. 2002, Lip et al. 2002, Wang et al. 2009) as well as in various animal experimental models of hypertension such as angiotensin II-induced hypertension (Wakui et al. 2013), endothelin-1-induced hypertension (Elmarakby et al. 2005), Dahl salt-induced hypertension (Zhou et al. 2006, Zicha et al. 2001), deoxycorticosterone acetate (DOCA)-salt induced hypertension (Gomez-Guzman et al. 2012, Viel et al. 2008), fructose-induced (Al-Awwadi et al. 2005, Korandji et al. 2011) or lead-induced hypertension (Vaziri and Sica 2004). Elevated ROS production was observed in the spontaneously hypertensive rats (Condezo-Hoyos et al. 2012, Slezak et al. 2014, Virdis et al. 2009). In addition, high BP per se was found to increase arterial superoxide production primarily by activating directly a NAD(P)H oxidase pathway, but also, in part, via activation of the local renin-angiotensin system (Ungvari et al. 2004).

Mechanism of ROS action was found to be associated also with the reduction of bioavailable NO (Vaziri and Sica 2004, Zhou et al. 2006) and altered local vascular mechanisms. In addition to superoxide, other ROS, such as hydrogen peroxide and hydroxyl anion, were shown to affect endothelial function and to modify vascular tone of the arteries depending on oxidative status (Puri et al. 2013). ROS were shown to activate cyclooxygenase concurrently with the inhibition of prostacyclin-mediated relaxation that results in accentuation of thromboxane A₂-induced constriction (Bachschmid et al. 2013, Korbecki et al. 2013). Furthermore, ROS inhibit vascular smooth muscle cell hyperpolarization and reduces H₂S-mediated relaxation, supporting the arterial constriction. The effect of ROS on vascular function may be accentuated after inhibition of NO production that may result in elevated bioavailability of superoxide (Testai et al. 2015).

In this study, we used an experimental model in which SNS, RAS and NO production were attenuated, although the influence of these systems may be still present in the minor extent. For example, under the conditions of pharmacological inhibition of adrenoreceptors, the co-transmitters such as ATP and NPY may still partially supply peripheral signal transmission (Racchi et al. 1999). Similarly, angiotensin II can be produced by angiotensin-converting enzyme-independent pathways that provide an alternative source of angiotensin II (Li et al. 2004). Furthermore, NO may be released from S-nitrosothiols to restore, at least partially, NO-dependent relaxation (Drobná et al. 2015, Maron et al. 2013). Moreover, lack of NO-dependent relaxation can be compensated by other endothelium-derived relaxing factors (Behuliak et al. 2011). These supplementary mechanisms can be effective in the regulation of BP after SNS, RAS and/or NOS inhibition and they can affect stress-induced BP responses as it was observed in this study in P+C+L-treated rats. In addition, this study showed that ROS-mediated mechanisms are involved in acute post-stress regulation of BP. As it is mentioned above, ROS were described as endothelium-derived contracting factors in various models of hypertension (Bernatova 2014), thus their local vasoconstricting effects may play a significant role in BP recovery under condition when SNS- and RAS-mediated mechanisms are inhibited.

In conclusion, our results showed that the lack of ROS resulted in greater stress-induced MAP decrease and prolongation of time required to reach new post-stress steady state in rats with temporally impaired blood pressure regulation. Thus, ROS-mediated mechanisms may participate in short-term local regulation of BP when main constrictor systems are temporarily ineffective. Our data suggest the involvement of local vascular mechanisms yet the exact signal pathways needs to be clarified in further studies.

Conflicts of Interest
There is no conflict of interest.

Acknowledgements
The authors thank Mrs. J. Petova and M. Majzunova, PhD. for their technical assistance. This study was...
References


