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Differential contributions of NOS isoforms in the rostral ventrolateral medulla to cardiovascular responses associated with mevinphos intoxication in the rat

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Abstract

The organophosphate poison mevinphos (Mev) elicits cardiovascular responses via nitric oxide (NO) produced on activation of M2 muscarinic receptors (M 2R) in the rostral ventrolateral medulla (RVLM), where sympathetic vasomotor tone originates. This study further evaluated the contribution of nitric oxide synthase (NOS) isoforms at the RVLM to this process, using adult Sprague–Dawley rats. Bilateral co-microinjection into the RVLM of the selective NOS I inhibitor (250 pmol), 7-nitroindazole or N ω-propyl-L-arginine antagonized the initial sympathoexcitatory cardiovascular responses to Mev (10 nmol). Co-administration of a selective NOS II inhibitor, N 6-(1-iminoethyl)-L-lysine (250 or 500 pmol) further enhanced these cardiovascular responses and reversed the secondary sympathoinhibitory actions of Mev. A potent NOS III inhibitor, N 5-(1-iminoethyl)-L-ornithine (46 or 92 nmol) was ineffective. We also found that M 2R co-localized only with NOS I- or NOS II-immunoreactive RVLM neurons. Furthermore, only NOS I or II in the ventrolateral medulla exhibited an elevation in mRNA or protein levels during the sympathoexcitatory phase, with further up-regulated synthesis of NOS II during the sympathoinhibitory phase of Mev intoxication. We conclude that whereas NOS III is not engaged, NO produced by NOS I and II in the RVLM plays, respectively, a sympathoexcitatory and sympathoinhibitory role in the cardiovascular responses during Mev intoxication.

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Keywords: Mevinphos intoxication; Nitric oxide synthases; Rostral ventrolateral medulla; Cardiovascular responses

1. Introduction

Recent years saw renewed interest in organophosphate poisons because of the impending threat of their deployment in chemical warfare or terrorist attacks (Balali-Mood and Shariat, 1998), along with gradual increase in accidental and suicidal poisoning because of easy access to organophosphate pesticides in agriculture and gardening (Bardin et al., 1994). As an inhibitor of cholinesterase (Tafuri and Roberts, 1987), it is generally contended that clinical presentations of organophosphate poisoning result from accumulation of, and over-stimulation by acetylcholine at peripheral and central synapses. Intravenous administration of various organophosphates induces hypertension in animals via cholinergic activation of the brain stem, which in turn elicits pressor responses by sympathoexcitation (Takahashi et al., 1991). Relatively little information is currently available, however, on the cellular and molecular mechanisms in the brain stem that are responsible for the cardiovascular manifestations during organophosphate intoxication.

Mevinphos (3-[dimethoxyphosphinyl-oxyl]-2-butenoic acid methyl ester; Mev) is the most commonly used organophosphate poison for suicidal purposes in Taiwan (Chuang et al., 1996). As an organophosphate of the P=O type, Mev exerts direct inhibition on acetylcholinesterase in the brain (Takahashi et al., 1991).
Patients who died of suicidal ingestion of organophosphates, including Mev, exhibit significantly diminished power density in the vasomotor components of their systemic arterial pressure (SAP) signals (Yen et al., 2000). As the origin of these “life and death” signals (Kuo et al., 1997a) and the medullary location of premotor sympathetic neurons that are responsible for the maintenance of vasomotor tone (Ross et al., 1984), the rostral ventrolateral medulla (RVLM) is a site via which Mev elicits cardiovascular responses (Chang et al., 2001; Yen et al., 2001).

Mev intoxication may result from nitric oxide (NO) produced on activation of the M2 subtype of muscarinic receptors (M2R) by the accumulated acetylcholine in the RVLM (Chang et al., 2001). Of the three isoforms of NOS so far identified, neuronal NOS (NOS I or nNOS) is expressed in neurons and glial cells; inducible NOS (NOS II or iNOS) is localized in macrophages, smooth muscle cells and glial cells; and endothelial NOS (NOS III or eNOS) is principally present in endothelia, platelets and cardiomyocytes (Forstermann et al., 1995). Our laboratory has shown that physiological regulation of vasomotor outflow by the endogenous NO at the RVLM is determined by a balance between sympathoexcitation and sympathoinhibition induced, respectively, by the tonically active NOS I and NOS II (Chan et al., 2001c), with minimal contribution from NOS III (Chan et al., 2003). Furthermore, whereas NOS I and II are present in RVLM neurons, NOS III is associated primarily with blood vessels (Chang et al., 2003).

It follows that NOS I, II or III may be differentially associated with the location of M2R in the RVLM. Furthermore, these NOS isoforms may contribute differentially to the cardiovascular responses that are associated with Mev intoxication by undergoing varying changes in molecular synthesis and functional expression. Both hypotheses were validated in the present study, based on complementary results from immunohistochemical, physiological, pharmacological and biochemical experiments.

2. Methods

2.1. Animals

One hundred and fifty one specific pathogen-free adult, male Sprague-Dawley rats (284–350 g) purchased from the Experimental Animal Center of the National Science Council, Taiwan, ROC were used. The experimental procedures conformed to the guidelines approved by the institutional committee on experimental animals. All efforts were made to minimize animal suffering, and to reduce the number of animals used.

2.2. Double immunofluorescence staining

Animals processed for double immunofluorescence staining for M2R and either NOS I, II or III were anesthetized with pentobarbital sodium (50 mg/kg, i.p.). The staining procedures were modified from those reported previously (Chang et al., 2001, 2003). In brief, free-floating sections of the medulla oblongata were incubated simultaneously with two primary antisera. These included a goat polyclonal anti-M2R antiserum (1:1000; SC-7472, Santa Cruz Biotechnology, Santa Cruz, CA, USA), together with either a rabbit polyclonal anti-NOS I (1:1000; SC-648, Santa Cruz), or anti-NOS III (1:1000; SC-654, Santa Cruz), or a goat polyclonal anti-NOS II (1:1000; SC-650-G, Santa Cruz) antiserum. The same sections were subsequently incubated concurrently with two appropriate secondary antisera (Molecular Probes, Eugene, OR, USA; 1:500). These included a rabbit anti-goat IgG conjugated with Alexa Fluor 568 (A11079) for M2R and a goat anti-rabbit IgG conjugated with Alexa Fluor 488 (A11034) for NOS I or III or a rabbit anti-goat IgG conjugated with Alexa Fluor 488 (A11078) for NOS II. Viewed under a Fluorview FV300 laser scanning confocal microscope (Olympus, Tokyo, Japan), immunoreactivity for M2R or NOS isoforms exhibited either red or green fluorescence when viewed individually, and yellow fluorescence indicates co-localization of M2R and NOS isoforms.

2.3. General preparation

Preparatory surgery for our physiological experiments (Chan et al., 2001a,b,c, 2002, 2003; Chang et al., 2001, 2003) was performed under an induction dose of pentobarbital sodium (50 mg/kg, i.p.), and included intubation of the trachea, and cannulation of a femoral artery or vein. Maintenance of anesthetic level was provided by intravenous infusion of propofol (Zeneca, Macclesfield, UK) at 30 mg/kg/h. We have shown previously (Yang et al., 1995a) that this scheme provided satisfactory anesthetic maintenance while preserving the capacity of central circulatory regulation. The head of the animal was fixed to a stereotaxic head-holder (Kopf 1430, Tujunga, CA, USA), and body temperature was maintained at 37 °C by a heating pad. During the experiment, animals were allowed to breathe spontaneously with room air.

2.4. Recording and power spectral analysis of SAP signals

The SAP signals were simultaneously subject to online and real-time power spectral analysis (Chan et al., 2001a,b,c; Chang et al., 2001, 2003). In brief, SAP signals sampled at a rate of 2048 Hz were truncated.
into 32-s (1024 points) time segments. For each time segment, our computer algorithm estimated the power density of the spectral components based on fast Fourier transform. It subsequently quantified the power of each component by calculating the area of power spectral density between two specified frequencies. By repeating these procedures continuously, we were able to examine the spectral changes of SAP signals on a real-time basis. We quantified the frequency components of the SAP signals by computing the power density of the very high-frequency (VHF; 5–9 Hz), high-frequency (HF; 0.8–2.4 Hz), low-frequency (LF; 0.25–0.8 Hz) or very low-frequency (VLF; 0–0.25 Hz) components was computed. The SAP spectra and power density of these four spectral components were displayed during the experiment, alongside pulsatile SAP, mean SAP (MSAP) and HR, in an on-line and real-time manner.

2.5. Microinjection of test agents into the RVLM

Bilateral microinjection of test agents into the RVLM (Chan et al., 2001a,b,c, 2002, 2003; Chang et al., 2001, 2003), at a volume of 50 nl, was carried out stereotaxically and sequentially with a 27-gauge needle that is connected to a 0.5-μl Hamilton microsyringe (Reno, NV, USA). The coordinates were: 4.5–5 mm posterior to lambda, 1.8–2.1 mm lateral to midline, and 8.1–8.4 mm below the dorsal surface of cerebellum. Test agents used were freshly prepared with artificial cerebrospinal fluid (aCSF) immediately before administration. Mev (kindly provided by Sinon Corporation, Taichung Country, Taiwan) was routinely given together with one of the test agents, which included two selective NOS I inhibitors (Ayajiki et al., 2001; El-Haddad et al., 2002), 7-nitroindazole (7-NI; RBI, Natik, NY, USA) or N5-(1-iminoethyl)-l-lysine (L-NIL; Tocris, Bristol, UK); or a potent NOS III inhibitor (Moore et al., 1994), N6-(1-iminoethyl)-l-arginine (L-NIO; RBI); a selective NOS II inhibitor (Moore et al., 1994), N5-(1-iminoethyl)-l-lysine (L-NIL; Tocris, Bristol, UK); or a potent NOS III inhibitor (Rees et al., 1990), N5-(1-iminoethyl)-l-ornithine (L-NIO; RBI). The doses used were the same as in our recent studies (Chan et al., 2001a, b, c; Chang et al., 2001, 2003) when these test agents were used for the same purpose as in the present study. Microinjection of aCSF served as the vehicle and volume control. The composition of aCSF was (mM): NaCl 117, NaHCO3 25, KCl 4.7, CaCl2 2.5, MgCl2 1.2, Na2HPO4 1.2 and glucose 11, pH 7.3–7.4. To avoid the confounding effects of drug interactions, each animal received only one pharmacological treatment.

2.6. Histology

At the conclusion of some physiological experiments, the animal was killed with an i.v. injection of saturated magnesium sulfate. The brain stem was removed after craniotomy and fixed in 30% sucrose in 10% formaldehyde–saline solution for at least 72 h. Histological verification of the microinjection site was carried out in 20-μm frozen sections stained with Neutral Red.

2.7. Tissue samples from ventrolateral medulla

For biochemical experiments, rats were perfused intracardially with 100 ml of warm (37 °C) saline that contains heparin (100 IU/ml). The brain stem was rapidly removed after craniotomy and placed on dry ice. Tissues on both sides of the ventrolateral part of medulla oblongata from each animal, at the level of the RVLM (0.5–2.5 mm rostral to the obex), were collected 30 or 180 min after bilateral microinjection into the RVLM of Mev (Chan et al., 2001a, b, c; Chang et al., 2003). All medullary samples thus obtained were stored at −80 °C and were pooled to provide sufficient tissues for either reverse transcription–polymerase chain reaction (RT–PCR) or western blot analysis (7–8 rats for either analysis).

2.8. Isolation of total RNA and RT–PCR

Isolation and extraction of total RNA from the ventrolateral medulla and RT–PCR analysis were carried out as reported previously (Chan et al., 2001a, b, c; Chang et al., 2003). The primers for NOS I and II were obtained from TIB Molbiol (Berlin, Germany), and those for NOS III and GAPDH were synthesized by Quality Systems (Taipei, Taiwan, ROC) based on sequences reported by Geerts et al. (1998) and Chang et al. (2003). The predominant cDNA amplification product predicted for NOS I, II, III or GAPDH was, respectively, 500, 513, 595 or 400 bp in length. The amount of mRNA products for NOS I, II or III was analyzed by the ImageMaster Video Documentation System (Amersham Pharmacia Biotech, Piscataway, NJ, USA), and was expressed as the ratio to GAPDH mRNA product.

2.9. Protein extraction and western blot analysis

Protein extraction and western blot analysis of proteins in the ventrolateral medulla were performed according to our reported procedures (Chan et al., 2001b; Chang et al., 2003). The primary antisera used included: rabbit polyclonal antiserum against NOS I (1:500; SC-648, Santa Cruz), NOS II (1:100; SC-650, Santa Cruz) or NOS III (1:100; SC-654, Santa Cruz) or a mouse monoclonal antiserum against β-actin (1:10,000; MAB1501, Chemicon, Temecula, CA). The secondary antisera (1:1000; Chemicon) used included: horseradish peroxidase-conjugated goat anti-rabbit (AP132A) or anti-mouse (AP124A) IgG. Specific
antibody–antigen complex was detected using an enhanced chemiluminescence western blot detection system (NEN Life Science Products, Boston, MA, USA). The amount of NOS I, II or III was quantified by the ImageMaster Video Documentation System (Amersham Pharmacia Biotech), and was expressed as the ratio to β-actin protein product.

2.10. Statistical analysis

All values are expressed as mean ± S.E.M. The averaged value of MSAP or HR calculated every 20 min after microinjection of test agents, or the sum total of power density for individual spectral components in the SAP spectra over 20 min, was used for statistical analysis. One-way or two-way ANOVA with repeated measures was used, as appropriate, to assess group means, followed by the Scheffé multiple range test for post hoc assessment of individual means. \( P < 0.05 \) was considered statistically significant.

3. Results

3.1. Differential co-localization of \( M_2R \) and NOS isoforms in the RVLM

We observed an extensive presence of \( M_2R \) immunoreactivity in cells throughout the confines of the RVLM (Fig. 1B,E,H). Intriguingly, a majority of RVLM cells that were immunoreactive to NOS I (Fig. 1A) or NOS II (Fig. 1D) also exhibited immunofluorescence staining for \( M_2R \) (Fig. 1C,F). On the other hand, NOS III (Fig. 1G,I) immunoreactivity was associated primarily with blood vessels (Chang et al., 2003). Preliminary results further indicated that RVLM cells that were immunoreactive to either \( M_2R \) (data not shown) or NOS I or II (Chang et al., 2003) were also positively stained with the neuronal marker, neuron-specific nuclear protein (NeuN).

3.2. Temporal effects of Mev on SAP, HR and power density of vasmotor components of SAP signals

As reported previously (Chang et al., 2001), bilateral co-microinjection into the RVLM of Mev (10 nmol) and aCSF resulted in a progressive decline in SAP that became significant 100 min postinjection, accompanied by a slow, albeit insignificant reduction in HR (Fig. 2). On-line power spectral analysis of SAP signals (Chang et al., 2001) further revealed that the sequence of cardiovascular responses to Mev may be divided into two phases (Figs. 3–5). Phase I Mev intoxication manifested a trend of augmented power density of the VHF, HF, LF or VLF components in the SAP spectrum. Phase II exhibited a return to baseline of the VHF and VLF power, and a further and significant reduction in the power density of HF and LF components of SAP signals.

3.3. Differential changes in NOS mRNA or protein levels in ventrolateral medulla during Mev intoxication

RT–PCR and western blot analysis (Fig. 6) showed that both NOS I and II in the ventrolateral medulla exhibited a significant increase in mRNA or protein levels during Phase I Mev intoxication. Whereas the molecular synthesis of NOS II was further up-regulated, NOS I expression returned to baseline during Phase II. On the other hand, NOS III mRNA or protein levels in the ventrolateral medulla remained essentially unaltered during both phases of Mev intoxication.

3.4. Differential effects of NOS inhibitors on Mev-induced cardiovascular responses

In the presence of the NOS I inhibitor, NPLA or 7-NI (250 pmol; Fig. 2A), the hypotension promoted by Mev (10 nmol) exhibited an early onset and was significantly augmented, alongside enhanced, albeit insignificant bradycardia. Bilateral co-microinjection into the RVLM of the NOS II inhibitor, L-NIL (250 or 500 pmol) significantly blunted the hypotension promoted by Mev, and the minor bradycardia was absent (Fig. 2B). On the other hand, effective doses of the NOS III inhibitor, L-NIO (46 or 92 nmol; Fig. 2C) did not elicit discernible antagonism on Mev-induced changes in SAP or HR.

The augmented power density of VHF, HF, LF or VLF component of SAP signals during Phase I Mev intoxication was significantly reduced on co-administration of NPLA or 7-NI into the RVLM (Fig. 3); but the HF or LF power was further enhanced on co-microinjection of L-NIL (Fig. 4). Interestingly, whereas both selective NOS I inhibitors elicited minimal effect (Fig. 3), the reduced power density of HF or LF spectral components during Phase II was significantly antagonized on co-administration of the selective NOS II inhibitor into the RVLM (Fig. 4). Co-application of L-NIO did not result in appreciable alterations in the phasic changes in the power density of all spectral components of SAP signals induced by Mev (Fig. 5).

3.5. Microinjection sites

Histological verification indicated that test agents were delivered to the RVLM. Microinjection of test agents into areas outside the confines of the RVLM elicited minimal effect on mRNA or protein levels of NOS isoforms, SAP, HR or power density of spectral components of SAP signals.
4. Discussion

The premotor sympathetic neurons in the RVLM are long considered to be essential to the maintenance of resting and reflex control of SAP (Ross et al., 1984). Our laboratory reported previously (Yen et al., 2001) that Mev acts directly on the RVLM as a cholinesterase inhibitor (Takahashi et al., 1991), and NO produced on activation of the M2R by the progressive accumulation of acetylcholine over time in this medullary site is intimately related to the phasic cardiovascular events over the course of Mev intoxication (Chang et al., 2001). The present study extended these findings to provide the first demonstration that whereas NO produced by NOS I and NOS II in the RVLM plays, respectively, a sympathoexcitatory and sympathoinhibitory role, NOS III is not engaged in this process.

Acetylcholine acts through M2R within the RVLM to elicit elevation in SAP (Kubo et al., 1997), and releasable pools of acetylcholine or M2R (Ernsberger et al., 1988) are present in this medullary site. Whereas low doses of carbachol or physostigmine induce
hypertension and tachycardia, high doses of the muscarinic agonist or cholinesterase inhibitor elicited hypotension and bradycardia (Willette et al., 1998). Immunohistochemical (Simonian and Herbison, 1996) or in situ hybridization (Iwase et al., 1998) studies demonstrated the presence of NOS in the RVLM. Muscarinic activation of the RVLM elicits antinociception via an arginine/NO/cyclic GMP cascade (Iwamoto and Marion, 1994). Muscarinic stimulation also reduces HR via activation of NO production (Hare and Colucci, 1995). However, a crucial premise for NOS I or II to play an active role in the cardiovascular responses that are associated with Mev intoxication is the presence of either NOS isoform in RVLM neurons that contain M2R. This premise is satisfied by our observation that M2R immunoreactivity co-localized with NOS I- or NOS II-immunoreactive cells in the RVLM. Together with our recent demonstration (Chang et al., 2003) that these two enzymes are present in neurons within the confines of the RVLM, our results provided the crucial link to the notion that the phasic changes in spectral components of SAP signals may reflect the time-course of direct inhibition of cholinesterase by Mev, the progressive accumulation of acetylcholine that acts on M2R, and subsequent production of NO by NOS I and II in the RVLM. Preliminary results also demonstrated that muscarinic blockade in the RVLM with atropine blunted the hemodynamic effects and molecular synthesis of NOS I and II induced by Mev.

Our laboratory proposed recently (Chan et al., 2001c) that under physiological conditions, both NOS I and NOS II in the RVLM are active at the levels of functional expression and molecular synthesis. In addition, the prevalence of NOS I over NOS II activity and the associated dominance of sympathoexcitation over sympathoinhibition may underlie the maintenance of sympathetic vasomotor outflow and stable SAP by the endogenous NO in the RVLM. We further postulated that a shift in this balance in favor of NOS II may account for the reduction in sympathetic vasomotor outflow from the RVLM seen during experimental endotoxemia (Chan et al., 2001a) or Mev intoxication (Chang et al., 2001). These operational schemes for NO generated by NOS I or II in the RVLM are again validated in the present study, based on our current interpretation of the physiological significance of the spectral components of SAP signals.

It is generally stipulated that the VHF component is a reasonable representation of myocardial performance (Yang et al., 1995b), and the HF component is generated by the respiratory pumping mechanism (Triedman...
subject to additional amplification by the β-adrenergic system on the heart (Kuo et al., 1996). Both LF and VLF components represent neurogenic vasomotor outflow to blood vessels (Yang et al., 1995b; Yien et al., 1997) that originates from the RVLM, the power of which is reduced by phentolamine or prazosin (Kuo et al., 1997a). Furthermore, the power of the LF component is related to the synchronizing influence of baroreceptor afferents (Cerutti et al., 1994), and the VLF component may arise from the RVLM.
non-oscillatory perturbations of regional vasculature (Julien et al., 1995).

Phase I Mev intoxication

With the presence of an effective baroreceptor regulatory machinery (LF) and enhanced respiratory drive (HF), the overall sympathoexcitatory action on the heart and blood vessels represented by the augmented VHF and VLF power during Phase I Mev intoxication is reflected only by a slow and insignificant hypotension and bradycardia (Chang et al., 2001; Yen et al., 2001). Our present results revealed that a balance between NO produced by NOS I and II in the RVLM plays a pivotal role in this phase of Mev intoxication. We observed a significant surge in mRNA and protein levels of both NOS I and II in the ventrolateral medulla. Intriguingly, the augmented power density of VHF, HF, LF or VLF component of SAP signals was significantly reduced by two selective NOS I inhibitors, but the HF or LF power was further enhanced by a selective NOS II inhibitor. In addition, whereas both NOS I inhibitors augmented the Mev-induced reduction in SAP, the NOS II inhibitor blunted the minor hypotension and bradycardia.

Phase II Mev intoxication

The return of VHF and VLF power to baseline levels during Phase II Mev intoxication signifies sufficient cardiac contractility and basal neurogenic vasomotor tone that originates from the RVLM. Nonetheless, the significantly reduced HF and LF power points to detrimental decline in respiratory drive and a weakening of the capacity to generate additional sympathetic vasomotor discharges to the blood vessels as a component of the baroreceptor reflex (Chang et al., 2001; Yen et al., 2001). Our results indicate that NO generated by NOS II in the RVLM may be a crucial mediator of these cardiovascular ramifications during this phase of Mev intoxication. We observed a progressive up-regulation of molecular synthesis of NOS II in the ventrolateral medulla, and blockade of NOS II activity in the RVLM essentially blunted the significant reduction in SAP and power density of HF or LF component. More importantly, these enhanced molecular synthesis and function expression of NOS II in the RVLM took place without the opposing actions of NOS I.

Several mechanisms may account for the differential actions or time-courses of NO generated in the RVLM by NOS I or II. Whereas small amounts of NO generated by NOS I in the RVLM promote sympathoexcitation via glutamatergic neurotransmission (Chan et al., 2003), large amounts of NO produced by NOS II elicit sympathoinhibition via GABAergic neurotransmission (Chan et al., 2003) or production of peroxynitrite (Chan et al., 2002). At the same time, whereas the sympathoexcitatory actions of endogenous NO generated by NOS I in the RVLM exhibit a pattern of fast onset and short duration; the prolonged sympathoinhibitory responses to NO induced by NOS II develop slowly (Chan et al., 2001c, 2003). Our experimental design does not allow us to decipher the inter-relationship between activation of M3R and induction of NOS I or II in the RVLM. We noted that M2R-mediated gener-
ation of NO in rat isolated aorta engages calcium/calcium-modulin and protein kinase C (Sterin-Borde et al., 1995). Interestingly, extracellular NO inhibits M2R functions in rat lungs (Golkar et al., 2000). These observations provide possible clues for further delineation of the interplay between M2R and NOS I or II in the RVLM during Mev intoxication.

The lack of association with M2R in the RVLM, together with minimal alteration in mRNA or protein levels of NOS III in the ventrolateral medulla and ineffectiveness of L-NIO to affect the cardiovascular events during Mev intoxication, suggest that NOS III at the RVLM may not be actively engaged in this process. We noted that NO derived from NOS I increases SAP in NOS III null mutant mice (Huang et al., 1995; Kurihara et al., 1998). We also are aware that massive production of NO in the RVLM by transfecting adenoviral vectors encoding NOS III (Kishi et al., 2001), similar to activation of NOS II by local application of endotoxin (Chan et al., 2001b, 2002), decreases SAP, HR, sympathetic nerve activity or power density of vaso-motor components of SAP signals. At the same time, activation of M3R and M4R results in marked activation of NOS III in Chinese hamster ovary cells (Waid et al., 2000). Thus, whether NOS III in the RVLM plays a role in cardiovascular regulation under pathological conditions other than Mev intoxication awaits further elucidation.

We are aware that the selectivity of our test agents may affect the interpretation of our results. In this regard, 7-NI is suggested to be selective against NOS I, and NPLA is one of the most selective NOS I inhibitors (Ayajiki et al., 2001; El-Haddad et al., 2002). That both 7-NI and NPLA showed similar potency to antagonize the cardiovascular responses induced by Mev during Phase I further ascertained their selectivity in antagonizing NOS I. L-NIL was reported (Moore et al., 1994) to be 28 times more potent and selective in inhibiting NOS II than NOS I; and L-NIO is an irreversible endothelial NOS inhibitor (Rees et al., 1990) that shows little selectivity for NOS I and NOS II (Moore et al., 1994).

In conclusion, the present results demonstrated the differential contributions of NOS isoforms at both mol-

Fig. 6. Representative gels of RT–PCR or western blot products (inset) or amount of NOS mRNA or protein relative to GAPDH mRNA or β-actin protein, detected from the ventrolateral medulla 30 (Phase I) or 180 (Phase II) min after animals received bilateral microinjection into RVLM of Mev (10 nmol). Lanes 1, 3 and 5 or filled bars: samples from animals that received microinjection of aCSF. Lanes 2, 4 and 6 or open bars: samples obtained during basal, Phase I or II Mev intoxication. Values are mean ± S.E.M. of triplicate analyses, n = 7–8 animals per experimental group. *P < 0.05 vs aCSF group in the Scheffé multiple-range test.
ecular synthesis and functional expression levels in the RVLM to the cardiovascular responses that are associated with Mev intoxication. Whereas NO produced by a surge in the production and activity of NOS I and II in the RVLM is responsible, respectively, for the sympathoexcitatory and sympathoinhibitory phase of Mev intoxication, NOS III does not appear to be engaged in this process. In a series of studies carried out in the intensive care unit, our laboratory identified previously a common denominator among patients who succumbed to either systemic inflammatory response syndrome (Yien et al., 1997), brain injury (Kuo et al., 1997b) or organophosphate poisoning (Yen et al., 2000); death is invariably preceded by a dramatic reduction or loss of the LF and VLF components of SAP signals. In the pursuit of mechanisms that underlie this potentially new clinical marker for “life and death”, we demonstrated in a rat model of experimental endotoxemia (Chan et al., 2001a), which mimics systemic inflammatory response syndrome, that the progression towards death is associated with the progressive augmentation in both molecular synthesis and functional expression of NOS II in the RVLM. It is intriguing that the present study revealed that up-regulated synthesis and activity of NOS II in the RVLM also subserve the “pro-death” phase of Mev intoxication. The identification of common biochemical and physiological mechanisms that underlie the death process therefore opens a new vista in our search for cellular and molecular mechanisms that are associated with fatal cardiovascular depression during organophosphate poisoning.

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