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Enhanced nitric oxide release/synthesis in the posterior hypothalamus during nitroglycerin tolerance in rats

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Abstract

We have recently observed that increasing central noradrenergic transmission and sympathomimetic activity is involved with the complex hemodynamic effects during tolerance to nitroglycerin. The present study was to examine the release of nitric oxide (NO) in the posterior hypothalamus during tolerance to depressor responses to nitroglycerin and determine if, during the tolerance, endogenous NO synthesis is induced in the posterior hypothalamus. A microdialysis probe was implanted in the posterior hypothalamus and perfusion fluid was pumped through the probe at 2 μl/min in conscious rats. Tolerance to nitroglycerin was produced by three intravenous (i.v.) injections of 1.3 mg nitroglycerin each within 40 min compared to the same administrations of low dose of the drug, sodium nitroprusside and papaverine. Dialysate samples were collected 1 h before and 1 h each after injections for 8 h. Concentrations of nitrite (NO_2^-), nitrate (NO_3^-), and total nitrite plus nitrate (NO_x^-) were quantified in the samples by using chemiluminescence. The dose–response curve for arterial depressor induced by intravenous injection of the challenge doses of nitroglycerin was markedly shifted to the right at the first hour after nitroglycerin tolerance, lasted 3 to 5 h and reversed at 7 h. The dialysate NO_3^- and NO_x^- concentrations in the posterior hypothalamus were significantly increased at the first hour following nitroglycerin tolerance but were not altered by low dose of the drug, sodium nitroprusside, and papaverine. Nitroglycerin tolerance predominantly caused an increase in NO_3^- release in the posterior hypothalamus with no or small amount of changes in dialysate NO_2^- and the response was partially inhibited by pretreatment with N^G-Propyl-L-arginine (NPLA) (1.0 mg/kg, i.p.), an inhibitor of neuronal NO synthesis. The increase of NO release in the posterior hypothalamus occurred at the first hour, lasted 2 to 3 h and reversed at 5 to 6 h during nitroglycerin tolerance. The results show that systemically administered high dose of nitroglycerin increases NO release in the posterior hypothalamus which matches the time interval of tolerance to arterial depressor response to the drug. Data suggest that there is an enhanced endogenous NO synthesis in the posterior hypothalamus which may affect central sympathetic functions during nitroglycerin tolerance.

Keywords: Nitroglycerin; Nitrate tolerance; Nitric oxide (NO); Posterior hypothalamus; Cardiovascular action; Dialysis

1. Introduction

Nitroglycerin has been one of the most widely used vasodilating drugs for more than a century (Needleman et al., 1985). The drug undergoes biotransformation to nitric oxide (NO), which then binds to and activates guanylate cyclase catalyzing production of cyclic GMP and initiating relaxation of vascular smooth muscle (Brien et al., 1987; Napoli and Ignarro, 2003). The efficacy of nitroglycerin is limited by the nitrate tolerance, which causes partial or complete attenuation of vasodilator effects (Needleman et al., 1985; Erhardt, 1986). Nitrate tolerance develops rapidly and may be reversed within 12 h (Parker, 1990; Silber, 1990; Rinde-Hoffman et al., 1991). The mechanism of tolerance to organic nitrates remains poorly defined and is still controversial. Previous studies by Ma and Long have demonstrated that nitroglycerin increases release and synthesis of norepinepherine in the brain, and it has been suggested that central noradrenergic transmissions contribute to tolerance and cardiovascular effects of the drug (Ma and Long, 1991, 1992; Ma et al., 1992, 1994).

It has been also demonstrated that intravenous nitroglycerin acts centrally to relieve cerebral vasospasm in both patients and animals (Kistler et al., 1979; Frazee et al., 1981). Systemically administered nitroglycerin inhibits reflex vasoconstriction of coronary vessels and the effect is

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reported to be mediated by increases in the release of norepinepherine in the central nervous system (Kaverina, 1960; Kaverina et al., 1967). We have recently demonstrated that acute tolerance induced by systemically administered nitroglycerin predominantly increases nitrite (NO_2^-) and nitrate (NO_3^-) concentrations in the posterior hypothalamus tissues but not in other brain regions (Ma et al., 1999). The hypothalamus region is one of the most important central areas for regulation of cardiovascular system and the posterior hypothalamus has been considered as a pressor area (Bousquet and Schwartz, 1983; Nakata et al., 1990; Shonis and Waldrop, 1993). Norepinephrine in the posterior hypothalamus contributes to increases in sympathetic nerve activity, arterial blood pressure, and heart rate while bilateral lesions of the posterior hypothalamus produce depressor effects (Nakata et al., 1990; Shonis and Waldrop, 1993).

Recent studies have shown that nitric oxide (NO) is perhaps one of the most important messenger molecules produced in many types of cells including neurons in the brain (Moncada and Higgs, 1991; Bredt and Snyder, 1992; Ma et al., 1995). NO may serve as a messenger for neurons, much like a neurotransmitter or a neuromodulator representing a widespread signaling mechanism and function (Garthwaite et al., 1988; Bredt and Snyder, 1992). Investigators have shown that nitroglycerin undergoes biotransformation to glyceryl-1,2- and 1,3-dinitrate and NO_2^-, which may be an initial step in the production of NO (Brien et al., 1986, 1987). Organic nitrates are lipophilic and readily cross biological membranes to generate exogenous NO in the brain (Needleman et al., 1985; Torfgard et al., 1989; Torfgard and Ahnler, 1991). Endogenous NO is synthesized from L-arginine by an enzymatic pathway. Analogues of L-arginine such as N^G-nitro-L-arginine methyl ester and N^G-nitro-L-arginine have been used widely as inhibitors of the synthesis of NO from L-arginine both in vivo and in vitro (Moncada and Higgs, 1991). N^G-Propyl-L-arginine (NPLA) has been shown to be a selective inhibitor of neuronal NO synthesis (Zhang et al., 1997). The chemical lability of NO in cells and tissues has been attributed to a rapid oxidation to both NO_2^- and NO_3^- (Ignarro, 1990; Ignarro et al., 1993; Kurz et al., 1993). In the living system, oxyhemoglobin and oxymyoglobin catalyze the complete conversion of NO or NO_2^- to NO_3^-, the stable metabolites (Ignarro et al., 1993). Thus, measurement of these two metabolites (NO_2^- and NO_3^-) has been considered a very adequate indicator of the presence of NO in the experiments with intact cells, tissues, and whole animals (Ignarro, 1990; Ignarro et al., 1993; Kurz et al., 1993).

The purpose of the present study was to quantify dialysate NO metabolites, NO_2^-, NO_3^-, and total nitrite and nitrate (NO_x^-) concentrations in the posterior hypothalamus following systemical administration of high dose of nitroglycerin compared to those induced by low dose of the drug, sodium nitroprusside, and papaverine, a pharmacological vasodilator. Influence of endogenous NO generation by systemically administered nitroglycerin will be tested by applying a selective inhibitor of NO synthesis. The correlation of NO release in the posterior hypothalamus and nitroglycerin tolerance will be examined by quantification of dialysate NO metabolites in the area combined with observation of arterial blood depressor responses to systemic administration of the drug.

2. Materials and methods

2.1. General

All experiments were performed using adult male (5–8 months) Sprague–Dawley rats. The protocol was approved by the Harbor-UCLA Animal Use Review Committee, and was in accordance with AAALAC and NIH guidelines. The animals were maintained on a 12-h light–dark cycle in temperature and humidity-controlled rooms. Food (Regular Rat Chow, Dyets) and water were available ad libitum.

2.2. Cardiovascular responses to nitrate tolerance

Rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.). Femoral venous cannulae were implanted for systemic delivery of anesthesia and compounds. A femoral arterial catheter was connected to the pressure transducer (Model p231D, Gould Electronics, Valley View, OH) for measurement of arterial blood pressure and heart rate on a Gould thermal array recorder (TA-4000, Gould Electronics). After recovery from surgery, simultaneous measurements of arterial blood pressure and heart rate were obtained and were allowed to stabilize for at least 20 min. Tolerance to nitroglycerin was produced by intravenous (i.v.) administration of 4 mg delivered as three pulse injections of 1.3 mg nitroglycerin within 40 min (Rush et al., 1971; Ma et al., 1999). Rats received i.v. injections of the challenge doses of nitroglycerin (3, 10, and 30 μg/kg) at 10 min, 1, 2, 3, 4, 5, 6, 7, and 8 h after the last time of injection of the high dose nitroglycerin (4 mg). Commercial nitroglycerin (solution contains alcohol 30%, propylene glycol 30%, and water) was diluted in physiological saline and intravenously administrated as each pulse injection. Drugs were given in a volume of 40 μl over a period of 30 s for low (challenge) doses and a volume of 250 μl over a period of 5 min for high (tolerance) doses. The dose–response curves for hypotensive responses to i.v. injection of nitroglycerin were obtained following treatment with high dose of the drug and at each time interval after nitrate tolerance.

2.3. Microdialysis in the posterior hypothalamus

Rats were anesthetized with sodium pentobarbital and were placed in a stereotaxic apparatus in a flat skull position. A CMA 12 Microdialysis Siliconized Guide (CMA/Microdialysis, North Chelmsford, MA) was implanted into the posterior hypothalamus using following coordinates: −4.0

posterior, +0.5 lateral and −8.3 ventral from bregma (Nakata et al., 1990). The guide cannula was secured in place with dental cement. At least 3 days after the surgery, the unanesthetized rat was gently wrapped in a towel and a CMA 12 dialysis probe, which has a 1-mm-long dialysis membrane (20 kDa cutoff), was implanted into the posterior hypothalamus through the guide cannula. After implanting the microdialysis probe, the rat was placed in a clear plastic hemisphere containing wood chips and allowed to stabilize for at least 2 h. The inlet/outlet dialysis capillaries were connected to polyethylene tubing. The inlet tube was connected to a 1-ml microsyringe driven by a CAM 102 Microdialysis Pump. The pump held 2, 1-ml syringes each with a different solution that could be switched to the inlet of the probe without stopping the pump. The outlet was positioned in a collecting tube set in a small box of ice. Artificial cerebral spinal fluid (pH adjusted to 7.4) was perfused at the rate of 2 μl/min (L'Heureux et al., 1986; Nakata et al., 1990). After a 90-min period of dialysis equilibration, two 30-min dialysate samples were collected for measurements of NO_2^-, NO_3^- and NO_x^- concentrations before and after various treatments. Following completion of the experiments, rats were sacrificed by sodium pentobarbital (150 mg/kg, i.v.) and the site of the dialysis tube was verified histologically on coronal and sagittal brain sections after cresyl violet staining.

2.4. Biochemical analysis of NO_2^-, NO_3^-, and NO_x^- concentrations

Concentrations of NO_2, NO_3^-, and NO_x^- in the dialysate samples were quantified by using a nitrogen oxide analyzer as described (Ignarro et al., 1993; Ma et al., 1999). Measurements of NO_2^-, NO_3^- and NO_x^- concentrations were conducted in a blind manner. Briefly, each sample was homogenized manually in a 1:10 ratio (tissue weight/volume of methanol). The mixture was centrifuged at 40,000 × *g* for 10 min in an Eppendorf 5415C microcentrifuge at 4 °C. A 60-μl aliquot of the supernatant was injected into a nitrogen oxide analyzer (Dasibi, Glendale, CA). Samples containing NO_2^- and NO_3^- were reduced to NO gas, which can be quantified by the chemiluminescence detection device after reaction with ozone. Refluxing 1% potassium iodide in glacial acetic acid causes a rapid one-electron reduction of NO_2^- to NO gas and this acidification/reduction solution was used to determine NO_2^- concentrations. NO_x^- concentrations were measured by using refluxing 1.5 mM vanadium (III) chloride in 2 M HCI. Values for NO_2^- and NO_x^- were quantified and values for NO_3^- were calculated by subtracting NO_2^- from NO_x^- values. The NO analyzer was calibrated with known concentrations of NO_2^- ($NaNO_2$). The standard curve was made each day, which encompassed the range of nitrogen oxides produced by the experimental samples. The quantitative analyses were based on measurements of peak areas of the standard compound ($NaNO_2$). The lower limit of the detection of this assay was 10 pmol of NO.

2.5. NO concentrations in the posterior hypothalamus responses to nitroglycerin and other vasodilators

Nitroglycerin (0.1, and 1.3 mg) or sodium nitroprusside (0.03 mg) was injected in rats as a single i.v. injection 15 min each for three times. The papaverine-treated group received three injections of the drug (negative control, 0.1 mg each), which decreased arterial blood pressure comparable to the levels achieved by injection of nitroglycerin (Imhof et al., 1989). A high (1.3 mg) dose of nitroglycerin was given in a volume of 250 μl over a period of 5 min and low doses of the drugs were injected in a volume of 40 μl over a period of 30 s. Two 30-min dialysate samples were collected before and after each injection with either nitroglycerin, sodium nitroprusside, papaverine or saline, as well as at 1, 2, and 3 h after the last injection during each subsequent perfusion condition. High dose of nitroglycerin-induced changes in dialysate NO_x^- concentrations in the posterior hypothalamus were also compared with the treatments using low dose of the drug, sodium nitroprusside, and papaverine at each subsequent perfusion condition. The responses to high dose (4 mg) of nitroglycerin-induced changes in dialysate NO_2^-, NO_3^- and NO_x^- concentrations in the posterior hypothalamus were also measured and compared at two 30-min intervals before (averaged to obtain a baseline value), and four 60-min intervals after the treatments.

2.6. Time responses to changes in NO concentrations in the posterior hypothalamus during nitrate tolerance and presence of an inhibitor of NO synthesis

Tolerance was induced by three intravenous injections of 1. 3 mg nitroglycerin each in a volume of 250 μl over a period of 5 min within a period of 40 min. In a group of rats, N^G-Propyl-L-arginine (NPLA, 1.0 mg/kg, i.p.) was administrated at 15 min before the first injection of nitroglycerin followed by three injections of the drug (Zhang et al., 1997; EL-Haddad et al., 2002). Two 30-min dialysate samples were collected at two 30-min intervals before injection (averaged to obtain a baseline value) and compared to two 30-min intervals after injection with either nitroglycerin or NPLA plus nitroglycerin, as well as at 1, 2, 3, 4, 5, 6, 7, and 8 h after the last time of injection during each subsequent perfusion condition. Dialysate samples were collected and NO_x^- concentrations in the samples were measured 1 h before and 1 h after each injection for 8 h with nitroglycerin alone compared to presence of NPLA. The time interval curves of changes in dialysate NO_x^- concentrations in the posterior hypothalamus were also compared to the curves of arterial pressure tolerance to nitroglycerin and the recovery periods.

2.7. Data presentation and statistical analysis

Results are expressed as means ± S.E. Mean arterial pressure is expressed as millimeters of mercury. Analysis of variance and student's *t*-test were used to analyze the

significant difference. P values less than 0.05 were considered significant.

2.8. Drugs and chemicals

The drugs and chemicals used in these experiments were nitroglycerin (Abbott), sodium nitroprusside (Abbott), papaverine (CIBA), and NPLA (Breon Laboratories). Compounds were diluted in physiological saline.

3. Results

3.1. Time responses to changes in arterial blood pressure responses to nitroglycerin during tolerance

Control mean arterial blood pressure and heart rate were 106 ± 4.5 mm Hg and 366 ± 16 beats/min, respectively ($n = 6$). Fig. 1 shows decreases in mean arterial blood pressure induced by intravenous injection of the challenge doses of nitroglycerin before and at 1, 3, 5, and 7 h after acute tolerance to nitroglycerin. Low doses of nitroglycerin (3, 10, and 30 μg/kg) produced a dose-dependent decrease in mean arterial blood pressure. Tolerance to nitroglycerin (4 mg) was manifested by a shift in the dose–response curve for depression of arterial blood pressure, as shown in Fig. 1. The dose–response curve for arterial pressure depression induced by intravenous injection of the challenge doses of nitroglycerin was markedly shifted to the right at the first hour after administration of a high dose of the drug. This evidence of tolerance to nitroglycerin lasted 3 to 5 h and reversed at 7 h after the injections (Fig. 1). These findings

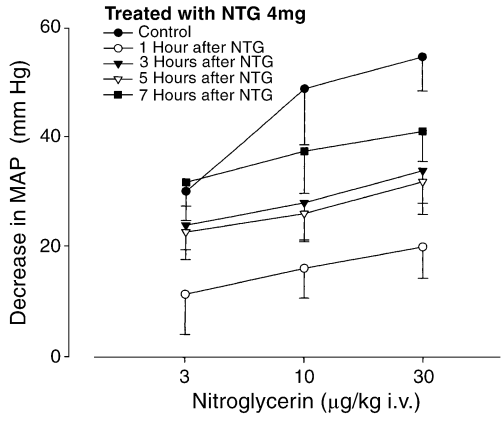

Fig. 1. Dose-dependent decreases in mean arterial pressure induced by intravenous administration of low doses of nitroglycerin before and at 1, 3, 5, and 7 h after three intravenous injections of 1.3 mg nitroglycerin each within 40 min in conscious rats. Depressor responses to challenge doses of nitroglycerin (3, 10, and 30 μg/kg) were markedly attenuated by high dose of nitroglycerin (4 mg) injection. The tolerance responses occurred at the first hour, lasted 3 and 5 h and reversed at 7 h after injection of high dose of nitroglycerin ($P < 0.05$, analysis of variance). Decreases in mean arterial blood pressure are shown as millimeters of mercury. Each point represents the mean values and S.E.M. ($n = 6$).

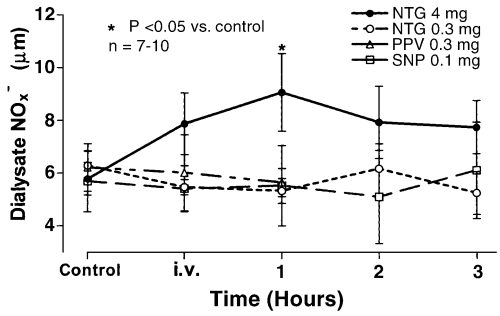

Fig. 2. Concentrations of dialysate nitrite plus nitrate (NO_x^-) in the posterior hypothalamus following three intravenous injections of 1.3 mg nitroglycerin (NTG, total 4 mg) within 40 min compared to three injections of low dose of nitroglycerin (0.1 mg each), papaverine (PPV, 0.1 mg each), and sodium nitroprusside (SNP, 0.03 mg each) in conscious rats. Dialysate NO_x^- concentrations were assayed 1 h before (control), 1 h during the three injections, and at 1, 2, and 3 h after injections of each compound. Dialysate NO_x concentrations in the posterior hypothalamus were significantly increased at the first hour after injections of 4 mg nitroglycerin. High doses of nitroglycerin revealed marginally significant differences in dialysate NO_x concentrations at the period of three pulse injections of the drug, and at 2 and 3 h after the treatment compared to control ($P = 0.06–0.1$). Each point represents the mean values of two 30-min dialysate samples and S.E.M. ($n = 7–10$). *: $P < 0.05$, compared with control.

are consistent with the results of previous studies of acute nitroglycerin tolerance in animals (Rush et al., 1971; Ma et al., 1999) and humans (Parker, 1990; Rinde-Hoffman et al., 1991).

3.2. Quantification of dialysate NO metabolites in the posterior hypothalamus following i.v. nitroglycerin and vasodilators

Fig. 2 shows dialysate NO_x^- concentrations in the posterior hypothalamus following systemic administration of high and low doses of nitroglycerin compared to sodium nitroprusside and papaverine. Dialysate NO_x^- concentrations in the posterior hypothalamus were significantly increased ($64 \pm 12\%$) at the first hour after 4 mg nitroglycerin injections compared to control but were not altered by low dose of the drug. Dialysate NO_x concentrations at the period of three pulse injections of high dose of nitroglycerin, and at 2 and 3 h after the treatment revealed marginally significant differences ($P = 0.06–0.1$) compared to control. Intravenous injections of sodium nitroprusside and papaverine did not change the dialysate NO_x^- concentrations in the posterior hypothalamus. Dialysate NO_x^- concentrations in the posterior hypothalamus were predominantly increased by systemically administered tolerance dose of nitroglycerin, but was not altered by low dose of the drug, sodium nitroprusside and papaverine (Fig. 2).

NO metabolites, NO_x^-, NO_2^-, and NO_3^- were measured in the posterior hypothalamus dialysate samples before and at 1, 2, and 3 h after acute tolerance to nitroglycerin. Fig. 3 shows the concentrations of dialysate NO_x^-, NO_2^-, and NO_3^- in the posterior hypothalamus following three intravenous

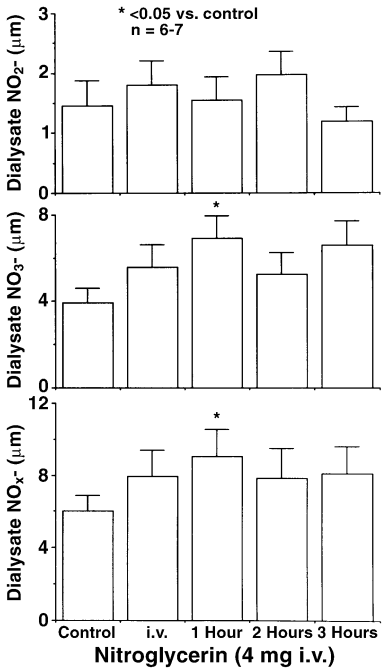

Fig. 3. Concentrations of dialysate nitrite (NO_2^-), nitrate (NO_3^-), and total NO_2^- plus NO_3^- (NO_x^-) in the posterior hypothalamus following three intravenous administrations of 1.3 mg nitroglycerin (nitroglycerin, total 4 mg). Dialysate NO_2^- (top), NO_3^-(middle), and NO_x^- (bottom) concentrations were collected and measured 1 h before (control), 1 h during the injections, and at 1, 2, and 3 h after injections of nitroglycerin. Each bar represents the mean values and vertical bars represent S.E.M. ($n=6-7$). *: $P<0.05$, compared with control.

injections of 1. 3 mg nitroglycerin each within 40 min. Intravenous high dose of nitroglycerin (4 mg) significantly increased dialysate NO_3^- and NO_x^- concentrations in the posterior hypothalamus at the first hour after injection (Fig. 3, middle and bottom panels). The levels of NO_3^- and NO_x^- concentrations in the posterior hypothalamus at 2 and 3 h after the treatment with nitroglycerin suggested an elevation, but did not produce statistically significant increase. NO_2^- concentrations in the posterior hypothalamus dialysate samples are relative low, about one third/fourth of NO_3^- concentrations both in control and treatment. Dialysate NO_2^- concentrations in the posterior hypothalamus were variable and were not altered following high dose of nitroglycerin, as shown in Fig. 3, top panels.

3.3. Time responses of dialysate NO metabolites in the posterior hypothalamus following nitrate tolerance and NO synthesis inhibition

The time intervals of NO release in the posterior hypothalamus were examined following nitroglycerin tolerance with or without presence of NPLA, an inhibitor of NO synthesis in rats. Fig. 4 displays the time–response curves of dialysate NO_x^- concentrations in the posterior hypothalamus following acute tolerance to nitroglycerin alone and in the presence of NPLA. Dialysate NO_x concentrations showed a significant increase over the time points following tolerance to nitroglycerin ($F_{5,70}=10.91$, $P<0.01$). The significant increase of dialysate NO_x^- concentration in the posterior hypothalamus occurred at the first hour, and reversed at 4 to 6 h after three intravenous injections of 1.3 mg nitroglycerin each within 40 min (Fig. 4). The levels of dialysate NO_x^- concentrations in the posterior hypothalamus at 2, and 3 h after 4 mg nitroglycerin treatment appeared to be increased, although the increases revealed marginally significant differences ($P=0.056$ and 0.06). The time intervals of enhanced NO release in the posterior hypothalamus are similar with arterial blood pressure tolerance to nitroglycerin, which reached a maximum at the first hour, lasted 3 to 5 h and reversed at 7 h after the injections (Figs. 1 and 4).

The effects of endogenous NO synthesis on the release of NO in the posterior hypothalamus were tested by pretreatment with NPLA before administration of the high dose of nitroglycerin. As shown in Fig. 4, dialysate NO_x concentrations in the posterior hypothalamus over all time points following pretreatment with NPLA were significantly lower than nitroglycerin treatment alone ($F_{5,70}=4.04$, $P<0.05$). Dialysate NO_x concentrations at 1, 2, and 3 h after pretreatment with NPLA revealed marginally significant differences compared to each level of time with nitroglycerin alone

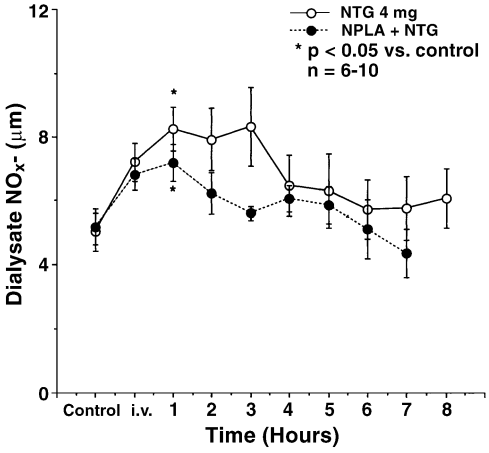

Fig. 4. The time responses to changes in dialysate nitrite plus nitrate (NO_x^-) in the posterior hypothalamus following three intravenous administrations of 1.3 mg nitroglycerin (NTG, total 4 mg) alone compared to pretreatment with N^G-Propyl-L-arginine (NPLA, 1.0 mg/kg, i.p.), an inhibitor of NO synthesis in conscious rats. Dialysate NO_x^- concentrations were assayed 1 h before (control), 1 h during the injections, and at 1, 2, 3, 4, 5, 6, 7, and 8 h after injections of nitroglycerin. Each point represents the mean values and S.E. ($n=6-10$). A two-way ANOVA revealed significant differences of dialysate NO_x concentrations over time points following nitroglycerin injections ($F_{5,70}=10.91$, $P<0.01$), as well as between the groups of pretreatment with NPLA and nitroglycerin alone over time points ($F_{5,70}=4.04$, $P<0.05$). Dialysate NO_x concentrations showed significant differences from control value at 1 h after treatment with nitroglycerin and NPLA plus nitroglycerin (*: $P<0.05$, Student's t-test). Pretreatment with NPLA revealed marginally significant differences in dialysate NO_x concentrations at 1, 2, and 3 h compared to each level of time in rats treated with nitroglycerin alone ($P=0.19$ at the first hour, 0.17 at the second hour, and 0.09 at the third hour by Student's t-test).

($P = 0.19$, 0.17, and 0.09, respectively). However, dialysate NO_x^- concentration in the posterior hypothalamus was significantly increased at the first hour by 4 mg nitroglycerin following pretreatment with NPLA. Systemically administered NPLA, an inhibitor of endogenous NO synthesis, did not fully block acute nitroglycerin tolerance-induced dialysate NO_x^- release in the posterior hypothalamus (Fig. 4).

4. Discussion

In the present study, treatment with tolerance dose of nitroglycerin resulted in a time-dependent enhancement of dialysate NO_3^- and NO_x^- concentrations in the posterior hypothalamus. The time interval of enhanced NO release in the posterior hypothalamus is similar with the periods of tolerance to arterial depressor responses to nitroglycerin. Nitroglycerin tolerance-induced dialysate NO_x^- increase in the posterior hypothalamus was partially inhibited by pretreatment with NPLA, a selective inhibitor of neuronal NO synthesis. It has been demonstrated that nitroglycerin undergoes biotransformation to NO, and is a potential exogenous source of NO (Brien et al., 1987; Napoli and Ignarro, 2003). Nitroglycerin and other organic nitrite and nitrate esters are lipophilic and readily enter brain and cells to be metabolized to NO. Subcutaneously administered nitroglycerin has been shown to accumulate in the brain, where it causes a marked increase in cGMP levels (Torfgard et al., 1989; Torfgard and Ahnler, 1991). Our previous results have demonstrated that systemically administered nitroglycerin causes predominant increases in NO metabolites in the posterior hypothalamus tissue (Ma et al., 1999). The results of the present study show that the dialysate NO_3^- and NO_x^- concentrations in the posterior hypothalamus were increased by high dose of nitroglycerin but were not altered by low dose of the drug. These findings, using the technique of posterior hypothalamus dialysis coupled to a time–response assay of arterial blood pressure tolerance to nitroglycerin, show that the time interval of enhanced NO release in the posterior hypothalamus is similar with arterial blood pressure tolerance to nitroglycerin, which reached the maximum at the first hour, lasted 3 to 5 h and reversed at 7 h. It has been demonstrated that nitrate tolerance develops rapidly and is reversed within 12 h (Parker, 1990; Silber, 1990; Rinde-Hoffman et al., 1991). Our findings are consistent with the time-responses results of the previous studies of acute nitroglycerin tolerance, and demonstrate that there is an enhanced NO release in the posterior hypothalamus during tolerance to depressor responses to nitroglycerin.

It has been generally accepted that nitroglycerin undergoes biotransformation to NO which stimulates guanylate cyclase to produce cyclic GMP that initiates relaxation of vascular smooth muscle (Brien et al., 1987; Napoli and Ignarro, 2003). However, investigators have demonstrated that nitroglycerin inhibits reflex vasoconstriction of coronary vessels and the effect is reported to be mediated by increases in release of norepinepherine in the central nervous system (Kaverina, 1960; Kaverina et al., 1967). Previous work by Ma and Long indicates that nitroglycerin increases release and enhances synthesis of norepinepherine at noradrenergic terminals in heart and brain (Ma and Long, 1991, 1992; Ma et al., 1992, 1994). Increasing central noradrenergic transmission and sympathomimetic activity correlates with the complex pharmacological effects of the drug (Ma et al., 1992, 1994). Intravenous repeated injections of low dose of nitroglycerin, sodium nitroprusside, an exogenous source of NO donor (Napoli and Ignarro, 2003), and papaverine, a pharmacological vasodilator (Imhof et al., 1989), did not change the concentrations of NO release in the posterior hypothalamus. These results suggest that neither biotransformation of NO from exogenous donors nor vasodilation-induced reflex activities contribute to NO release in the posterior hypothalamus. Nitroglycerin tolerance-induced NO release in the posterior hypothalamus was not fully inhibited by a selective inhibitor of neuronal NO synthesis. Thus, it appears that enhanced NO release in the posterior hypothalamus following nitroglycerin tolerance is partially involved with an increase in endogenous NO synthesis, which responds to repeated exposure of high dose of the drug.

It has been proposed that nitroglycerin is predominantly transformed intracellularly to produce NO by enzymatic mechanisms requiring three electron reduction and utilizing thiol-containing compounds such as cysteine and *N*-acetylcysteine (Ignarro et al., 1981; Kurz et al., 1993). Brien et al. (1986) have demonstrated that biotransformation of nitroglycerin occurs in vascular smooth muscle to glyceryl-1,2- and 1,3-dinitrate (GDN) and NO_2^-. It has been proposed that release of NO_2^- may be an initial step in the production of NO from nitroglycerin since NO_2^- is converted via nitrons acid (HONO) to NO. However, other experimental evidence demonstrates that the release of NO from nitroglycerin is not related to the formation of NO_2^-, but instead NO_2^- is associated with a degradative pathway for nitroglycerin (Kurz et al., 1993). The results of the present study show that dialysate NO_3^- in the posterior hypothalamus was three- to four-fold of those NO_2^- concentrations in control rats while systemically administered nitroglycerin predominantly caused an increase in NO_3^- release in the posterior hypothalamus with no or small amount of changes in dialysate NO_2^-. The chemical lability of NO has been attributed to a rapid oxidation to both NO_2^-/NO_3^- and NO_2^- is completely converted to NO_3^- catalyzed by oxyhemoglobin and oxymyoglobin in the living system (Ignarro, 1990; Ignarro et al., 1993). Thus, it is possible that enhanced NO_3^- release in the posterior hypothalamus might be generated from endogenous NO synthesis induced by systemically administered nitroglycerin. Recent studies have demonstrated that peripheral nitroglycerin administration induces co-localization of Fos expression with NADPH-diaphorase activity in hypothalamic neurons, which suggest a possible involvement of endogenous NO synthesis in

mediating the central responses to nitroglycerin (Tassorelli and Joseph, 1995a,b). Our findings would be consistent with this possibility, but we cannot exclude increases of NO release in the posterior hypothalamus via the cerebral circulation or crossing the blood–brain barrier following systemically administered nitroglycerin. More experiments would be required to address this issue. Despite this limitation, our chemical assay results agree with the pharmacological study by using an inhibitor of NO synthesis, and consistently suggest that there is an enhanced endogenous NO synthesis, which contributes to, at least in part, increased NO release in the posterior hypothalamus during nitroglycerin tolerance.

The development of tolerance to nitrates during repeated exposure of humans to these drugs was noted as early as the 19th century and continues to be reported (Needleman et al., 1985; Axelsson et al., 1986; Parker, 1990; Silber, 1990; Rinde-Hoffman et al., 1991). The mechanism of tolerance to organic nitrates and nitrites is unknown and may involve both neurohormonal adjustments and impairment of nitroglycerin metabolism at intracellular/subcellular processes (Needleman and Johnson, 1973; Axelsson et al., 1986; Parker and Parker, 1992; Boesgard et al., 1994; Chen et al., 1999). Results from in vitro studies suggest that the primary mechanism underlying vascular tolerance has been a depletion of vascular thiol compounds necessary for the bioconversion of organic nitrates (Needleman and Johnson, 1973). However, other studies indicate that hemodynamic tolerance to nitroglycerin occurs without changes in arterial and venous thiol levels; thus thiol depletion is apparently not the "cause" of nitrate tolerance in vivo (Boesgard et al., 1994). Our recent results demonstrate that the arterial pressure tolerance to nitroglycerin can be reversed by blockade of sympathetic function, and central NO production in the posterior hypothalamus tissues is enhanced by systemically administered nitroglycerin (Ma et al., 1999). The present studies show that systemically administered high dose of nitroglycerin causes increases in NO release/synthesis in the posterior hypothalamus, an important autonomic regulation site in the brain. It has been well-documented that posterior hypothalamus is a pressor area for central regulation of cardiovascular system, and norepinepherine in the posterior hypothalamus causes increases in sympathetic nerve activity, arterial blood pressure, and heart rate (Bousquet and Schwartz, 1983; Nakata et al., 1990; Shonis and Waldrop, 1993). Coexistence of NADPHd/nNOS and tyrosine hydroxylase in the brain nuclei has been demonstrated (Panzica et al., 1996; Simonian and Herbison, 1996) and suggests that noradrenergic neurons are capable of generating NO for regulation of noradrenergic activity within the brain in rats (Ye et al., 1997). It has been demonstrated that the use of converting enzyme inhibitors with nitroglycerin in patients prevented the development of tolerance (Imhof et al., 1989; Katz et al., 1991; Muiesan et al., 1991). Nitrate therapy is associated with an increase in circulating catecholamines and plasma renin activity is increased during continuous nitroglycerin therapy but not during intermittent treatment (Imhof et al., 1989; Dupuis et al., 1990; Muiesan et al., 1991; Parker and Parker, 1992). Moreover, it has been suggested that altered sympathetic compensatory reflexes may respond to nitrate tolerance since acute tolerance produced by nitroglycerin is prevented by pretreatment with guanethidine (Rush et al., 1971; Ma et al., 1999). The results of the present study support the previous findings of the sympathetic function responses to the complex hemodynamic effects of nitrate tolerance and further suggest that enhanced NO release/synthesis in the posterior hypothalamus may affect central sympathetic functions during nitroglycerin tolerance.

In summary, dialysate NO_3^- and NO_x^- in the posterior hypothalamus were increased by a systemically administered tolerance dose of nitroglycerin but not altered by a low dose of the drug, sodium nitroprusside, and papaverine. The time interval of nitroglycerin tolerance-induced NO release in the posterior hypothalamus is similar with the periods of tolerance to depressor responses to nitroglycerin. Nitroglycerin tolerance predominantly caused an increase in NO_3^- release in the posterior hypothalamus with no or small amount of changes in dialysate NO_2^- while nitroglycerin tolerance-induced dialysate NO_x^- in the posterior hypothalamus was partially inhibited by pretreatment with an inhibitor of neuronal NO synthesis. Thus, nitroglycerin tolerance is associated with an increase of NO release in the posterior hypothalamus which is involved with an enhanced endogenous NO synthesis. The results suggest that, during nitrate tolerance, there is an enhanced NO release/synthesis in the posterior hypothalamus which plays a potentially important role in the regulation of central sympathetic functions.

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References

Axelsson, K.L., Ahlner, J., Ljusegren, M.E., 1986. Tolerance to organic nitroesters in experimental animals. An assessment of in vitro and in vivo investigations. Acta Pharm. Toxicol. 59 (Suppl. VI), 121–128.

Bredt, D.S., Snyder, S.H., 1992. Nitric oxide—a novel neuronal messenger. Neuron 8, 3–11.

Brien, J.F., McLaughlin, B.E., Breedon, T.H., Bennett, B.M., Nakatsu, K., Marks, G.S., 1986. Biotransformation of glyceryl trinitrate occurs concurrently with relaxation of rabbit aorta. J. Pharmacol. Exp. Ther. 237, 608–614.

Brien, J.F., McLaughlin, B.E., Kobus, S.M., Kawamoto, J.H., Nakatsu, K., Marks, G.S., 1987. Mechanism of glyceryl trinitrate-induced vasodila-

tion. Relationship between drug biotransformation, tissue cyclic GMP elevation and relaxation of rabbit aorta. J. Pharmacol. Exp. Ther. 244, 322–327.

Boesgard, S., Aldershvile, J., Poulsen, H.E., Loft, S., Anderson, M.E., Meister, A., 1994. Nitrate tolerance in vivo is not associated with depletion of arterial or venous thiol levels. Circ. Res. 74, 115–120.

Bousquet, P., Schwartz, J., 1983. Alpha-adrenergic drugs: pharmacological tools for the study of the central vasomotor control. Biochem. Pharmacol. 32, 1459–1965.

Chen, J., Zhang, J., Stamler, J.S., 1999. Identification of the enzymatic mechanism of nitroglycerin bioactivation. Proc. Natl. Acad. Sci. 12, 8309–8311.

Dupuis, J., Lalonde, G., Lemieux, R., Rouleau, J.L., 1990. Tolerance to intravenous nitroglycerin in patients with congestive heart failure: role of increased intravascular volume, neurohormonal activation and lack of prevention with *N*-acetylcysteine. J. Am. Coll. Cardiol. 19, 923–931.

EL-Haddad, M.A., Chao, C.R., Ma, S.X., Ross, M.G., 2002. Neuronal nitric oxide modulates spontaneous and angiotensin II stimulated fetal swallowing behavior in the near term ovine fetus. Am. J. Physiol. 282, R1521–R1527.

Erhardt, L., 1986. Nitrate tolerance. State of the art-Chairman's summary. Acta Pharm. Toxicol. 59 (Suppl. VI), 129–132.

Frazee, J.G., Giannotta, S.L., Stern, W.E., 1981. Intravenous nitroglycerin for the treatment of chronic cerebral vasoconstriction in the primate. J. Neurosurg. 55, 865–868.

Garthwaite, J., Charles, S.L., Chess-Williams, R., 1988. Endothelium-derived relaxing factor release on activation of NMDA receptors suggests role as intercellular messenger in the brain. Nature 336, 385–388.

Ignarro, L.J., 1990. Biosynthesis and metabolism of endothelium-derived nitric oxide. Annu. Rev. Pharmacol. Toxicol. 30, 535–560.

Ignarro, L.J., Lippton, H., Edwards, J.C., Baricos, W.H., Hyman, A.L., Kadowitz, P.J., Gruetter, C.A., 1981. Mechanism of vascular smooth muscle relaxation by organic nitrates, nitrites, nitroprusside and nitric oxide: evidence for the involvement of *S*-nitrosothiols as active intermediates. J. Pharmacol. Exp. Ther. 218, 739–749.

Ignarro, L.J., Fukuto, J.M., Griscavage, J.M., Rogers, N.E., Byrns, R.E., 1993. Oxidation of nitric oxide in aqueous solution to nitrite but not nitrate: comparison with enzymatically formed nitric oxide from L-arginine. Proc. Natl. Acad. Sci. 90, 8103–8107.

Imhof, P.R., Rennwald, D., Muller, P., Howald, H., Burkart, F., 1989. Circulatory counter-regulations induced by continuous administration of nitroglycerin. Methods Find Exp. Clin. Pharmacol. 11, 409–414.

Katz, R.J., Levy, W.S., Buff, L., Wasserman, A.G., 1991. Prevention of nitrate tolerance with angiotensin converting enzyme inhibitors. Circulation 83, 1271–1277.

Kaverina, N.V., 1960. The mechanism of nitroglycerin action on the cardiac vessels. Bull. Eksp. Biol. Med. 49, 75–78.

Kaverina, N.V., Vysotskaya, N.B., Rosonov, Yu.B., Shugina, T.M., 1967. Connection between central action of glyceryl trinitrate and monoamine metabolism of the brain. Bull. Exp. Biol. Med. 64, 1310–1313.

Kistler, J.P., Lees, R.S., Candia, G., 1979. Intravenous nitroglycerin in experimental cerebral vasospasm: a preliminary report. Stroke 10, 26–29.

Kurz, M.A., Boyer, T.D., Whalen, R., Peterson, T.E., Harrison, D.G., 1993. Nitroglycerin metabolism in vascular tissue: role of glutathione *S*-transferases and relationship between NO and NO_2-formation. Biochem. J. 292, 545–550.

L'Heureux, R., Dennis, T., Curet, O., Scatton, B., 1986. Measurement of endogenous noradrenaline release in the rat cerebral cortex in vivo by transcortical dialysis: effects of drugs affecting noradrenergic transmission. J. Neurochem. 46, 1794–1801.

Ma, S.X., Long, J.P., 1991. Central noradrenergic activity is responsible for nitroglycerin-induced cardiovascular effects in the nucleus tractus solitarii. Brain Res. 559, 297–303.

Ma, S.X., Long, J.P., 1992. Central noradrenergic activity and the cardiovascular effects of nitroglycerin and amyl nitrite. J. Cardiovasc. Pharmacol. 20, 826–836.

Ma, S.X., Long, J.P., Bhatnagar, R.K., 1992. Further studies on central actions of nitroglycerin and lack of evidence for nitroglycerin interacting on [^{3}H]clonidine binding sites in cortex membranes. J. Pharmacol. Exp. Ther. 261, 1187–1194.

Ma, S.X., Schmid, P.G., Long, J.P., 1994. Noradrenergic mechanisms and the cardiovascular actions of nitroglycerin. Life Sci. 55, 1595–1603.

Ma, S.X., Abboud, F.M., Felder, R.B., 1995. Effects of L-arginine-derived nitric oxide synthesis on neuronal activity in nucleus tractus solitarius Am. J. Physiol. 268 . Regul. Integrative Comp. Physiol. 37, R416–R487.

Ma, S.X., Ignarro, L.J., Byrns, R., Li, X.Y., 1999. Increased nitric oxide concentrations in posterior hypothalamus and central sympathetic function on nitrate tolerance following subcutaneous nitroglycerin. Nitric Oxide: Biol. Chem. 3, 153–161.

Moncada, S., Higgs, E.A., 1991. Endogenous nitric oxide: physiology, pathology and clinical relevance. Eur. J. Clin. Investig. 21, 361–374.

Muiesan, M.L., Boni, E., Cefis, M., Cerri, B., Verdecchia, P., Porcellati, C., Agabiti-Rosei, E., 1991. Efficacy of transdermal nitroglycerin in combination with an ACE-inhibitor in patients with chronic stable angina pectoris. J. Am. Coll. Cardiol. 2 (Suppl A), 377A.

Nakata, T., Berard, W., Kogosov, E., Alexander, N., 1990. Microdialysis in the posterior hypothalamus: sodium chloride affects norepinephrine release, mean arterial pressure, heart rate and behavior in awake rats. Brain Res. Bull. 25, 593–598.

Napoli, C., Ignarro, L.J., 2003. Nitric oxide-releasing drugs. Annu. Rev. Pharmacol. Toxicol. 43, 97–123.

Needleman, P.J., Johnson, E.M., 1973. Mechanism of tolerance development to organic nitrates. J. Pharmacol. Exp. Ther. 184, 709–715.

Needleman, P., Corr, P.B., Johnson, E.M., 1985. Drugs used for the treatment of angina: organic nitrates, calcium channel blockers, and β-adrenergic antagonists. In: Gilman, A.G., Goodman, L.S., Rall, T.W., Murad, F. (Eds.), The Pharmacological Basis of Therapeutics. Macmillan, New York, pp. 806–826.

Panzica, G.C., Garzino, A., Ojeda, E.G., 1996. Coexistence of NADPH-diaphorase and tyrosine hydroxylase in the mesencephalic catecholaminergic system of the Japanese quail. J. Chem. Neuroanat. 11, 37–47.

Parker, J.O., 1990. Nitrate tolerance: a problem during continuous nitrate administration. Eur. J. Clin. Pharmacol. 38 (Suppl. 1), S21–S25.

Parker, J.O., Parker, J.D., 1992. Neurohormonal activation during nitrate therapy: a possible mechanism for tolerance. Am. J. Cardiol. 70, 93B–97B.

Rinde-Hoffman, D., Glasser, S.P., Arnett, D.K., 1991. Update on nitrate therapy. J. Clin. Pharmacol. 31, 697–701.

Rush, M.L., Lang, W.J., Rand, M.J., 1971. Studies on compensatory reflexes and tolerance to glyceryl trinitrate (GTN). Eur. J. Pharmacol. 16, 148–155.

Shonis, C.A., Waldrop, T.G., 1993. Augmented neuronal activity in the hypothalamus of spontaneously hypertensive rats. Brain Res. Bull. 30, 45–51.

Silber, S., 1990. Nitrates: Why and how should they be used? Current status of the clinical usefulness of nitroglycerin, isosorbide dinitrate and isosorbide-5-mononitrate. Eur. J. Clin. Pharmacol. 38 (Suppl. 1), S35–S51.

Simonian, S.X., Herbison, A.E., 1996. Localization of neuronal nitric oxide synthase-immunoreactivity within sub-populations of noradrenergic A1 and A2 neurons in the rat. Brain Res. 732, 247–252.

Tassorelli, C., Joseph, S.A., 1995a. Nitroglycerin induces Fos immunoreactivity in brainstem and forebrain structures of the rat. Brain Res. 682, 167–181.

Tassorelli, C., Joseph, S.A., 1995b. NADPH-diaphorase activity and Fos expression in brain nuclei following nitroglycerin administration. Brain Res. 695, 37–44.

Torfgard, K., Ahnler, J., 1991. Tissue levels of glyceryl trinitrate and cGMP

after in vivo administration in rat, and the effect of tolerance development. Can. J. Physiol. Pharm. 69, 1257–1261.

Torfgard, K., Ahnler, J., Axelsson, K.L., Norlander, B., Bertler, A., 1989. Tissue distribution of glyceryl trinitrate and the effect on cGMP levels in rat. Pharmacol. Toxicol. 64, 369–372.

Ye, S., Nosrati, S., Campese, V.M., 1997. Nitric oxide (NO) modulates the neurogenic control of blood pressure in rats with chronic renal failure (CRF). J. Clin. Invest. 99, 540–548.

Zhang, H.Q., Fast, W., Marletta, M.A., Martasek, P., Silverman, R.B., 1997. Potent and selective inhibition of neuronal nitric oxide synthase by *N*-Propyl-L-arginine. J. Med. Chem. 40, 3869–3870.