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Role of Ubiquitin-Proteasome System at Rostral Ventrolateral Medulla in Experimental Brain Stem Death

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Introduction

Life and death has been a phenomenon that has intrigued people of all walks of life since time immemorial. In the classical definition (Morison, 1971; Bernat et al., 1981; Youngner, 1992), the loss of heart and lung functions is sufficient to diagnose death. The advent of effective artificial cardiopulmonary support has necessitated a re-evaluation of this definition. It is now rather established that brain stem death is the physiological kernel of brain death, the anatomical substrate of its cardinal signs, and the main determinant of its invariable prognosis: asystole within hours or days (Mohandas and Chou, 1971; Pallis, 1983; Halevy and Brody, 1993; Wijdicks, 1995). Thus, the current legal definition of death in Taiwan, as in most developed countries, is brain stem death (Hung and Chen, 1995).

As much as brain stem death is a phenomenon of paramount medical importance, there is a dearth of information on its mechanistic underpinnings. Clinical studies during the 1990’s from our laboratory (Yang et al., 1995b; Yien et al., 1997; Kuo et al., 1997b; Yen et al., 2000) opened a new vista for mechanistic delineation of brain stem death. We identified a common denominator in comatose patients who succumbed to systemic inflammatory response syndrome (Yien et al., 1997), organophosphate poisoning (Yen et al., 2000) or brain injury (Kuo et al., 1997b). Death is invariably preceded by a dramatic reduction or loss of the low frequency components (0.004 to 0.15 Hz) in the power spectrum of their systemic arterial pressure (SAP) signals. Data obtained from brain death patients further suggest that this “life-and-death” signal is related to the functional integrity of the brain stem. Subsequent animal studies (Kuo et al., 1997a) revealed that the origin of this “life-and-death” signal resides in the rostral ventrolateral medulla (RVLM), where premotor neurons in the medulla oblongata that are responsible for maintaining stable sympathetic vasomotor outflows and blood pressure are located (Ross et al., 1984). We thus have in our hands a suitable neural substrate for evaluating the cellular and molecular basis of brain stem death. Since death represents the end of existence for an individual, the fundamental conceptual backbone in our pursuit for cellular and molecular mechanisms that underlie brain stem death is that the progression towards death entails multiple interacting “pro-death” and “pro-life” programs in the RVLM. It follows that evaluation of biochemical changes in the RVLM, whose neuronal activity is reflected in the waxing and waning of the “life-and-death” signal, should shed light on these programs.
The ubiquitin-proteasome system has emerged in recent years as a central player in the regulation of diverse cellular processes under physiological and pathological conditions. In addition to degradation of abnormal proteins, the ubiquitin-proteasome system is responsible for the recognition and degradation of ubiquitinated substrates such as proteins involved in cell cycle regulation, transcriptional regulation, receptor function, signal transduction, endocytosis, antigen presentation, aging, stress responses or apoptosis (Hershko and Ciechanover, 1992; Coux et al., 1996; Hershko and Ciechanover, 1998; Voges et al., 1999; Boraldi et al., 2003). Aberrations in either the process of ubiquitination or de-ubiquitination have also been directly implicated in the etiology of many diseases (Hilt and Wolf, 1996; Ciechanover, 1998; Schwartz and Ciechanover, 1999; Glickman and Ciechanover, 2002), including cancer (Maki et al., 1996; Spataro et al., 1998) and cardiomyopathy (Weekes et al., 2003).

One of the best-known targets of the ubiquitin-proteasome system is activation of the ubiquitous inducible transcription factor nuclear factor-κB (NF-κB) (Baeuerle and Baltimore, 1996; Whiteside et al., 1997; Yaron et al., 1997; Karin and Ben-Neriah, 2000). NF-κB is a heterodimeric complex consisting of a p50 subunit and a p65 subunit. It is retained in a latent form in the cytoplasm of non-stimulated cells by inhibitory molecules collectively termed IκB. Stimuli that induce NF-κB activation target the IκBs to degradation via a phosphorylation-regulated process. This site-specific phosphorylation directs the inhibitors to degradation via the ubiquitin-proteasome system. Following IκB degradation, NF-κB is translocated to the nucleus as an active factor that is able to induce its target genes. In this regard, it is of note that NF-κB-binding sites are present in the rat (Keinanen et al., 1999) or mouse (Wei et al., 2004) NOS II promoter, and are considered to be critical for gene expression. NF-κB is crucial for NOS II expression in human epithelial cells in response to pro-inflammatory cytokines and oxidants (Barnes, 1995). Thus, NOS II (Baeuerle & Henkel, 1994; Nathan et al., 1994; Xie et al., 1994; Keinanen et al., 1999; Piaggio et al., 2001; Pingle et al., 2003; Wei et al., 2004) genes are potentially regulated transcriptionally by NF-κB. On the other hand, scattered reports in the literature suggest that the ubiquitin-proteasome system may also be involved in the degradation of NOS II. For example, Musial and Eissa (2001) identified in human epithelial kidney HEK293 cells and murine macrophage cell line RAW 264.7 that the 26S proteasome is the primary degradation site for NOS II. This same group (Kolodziejski et al., 2002) further showed that ubiquitination is required for this degradative process. Ying and Sanders (2003) demonstrated the physiological importance of ubiquitination and proteasome
degradation of NOS II in the Dahl/Rapp salt-sensitive rats.

Previous studies from our laboratory revealed that reduction in the “life-and-death” signal during the progression towards brain stem death is associated with the progressive augmentation in both molecular synthesis and functional expression of NOS II (Chan et al., 2001a,b, 2003b, 2004a,b, 2007c). We further showed that transcriptional regulation of NOS II gene expression by NF-κB takes place in the RVLM (Chan et al., 2004a, 2007c). It follows that the ubiquitin-proteasome system may participate actively in brain stem death by regulating the synthesis and degradation of NOS II in the RVLM. Based on an experimental endotoxemia model of brain stem death, this hypothesis is validated.

Methods

General preparation. Experiments were carried out in male, adult Sprague-Dawley rats, and were in accordance with the guidelines for animal experimentation endorsed by our institutional animal care committee. Under initial pentobarbital sodium anesthesia (50 mg/kg, i.p.), the trachea was intubated and the right femoral artery and both femoral veins were cannulated. Animals received thereafter i.v. infusion of propofol (Zeneca) at 25 mg/kg/h. This scheme provides satisfactory anesthetic maintenance while preserving the capacity of central cardiovascular regulation (Yang et al., 1995a). The head of animals was fixed to a stereotaxic headholder (Kopf), and body temperature was maintained at 37°C with a heating pad. Animals were allowed to breathe spontaneously with room air during the experiment.

Recording and power spectral analysis of systemic arterial pressure signals. SAP signals recorded from the femoral artery were subject to on-line and real-time power spectral analysis (Chan et al., 2001a,b, 2002, 2003a-c, 2004a,b, 2005a,c, 2007a-c; Li et al., 2001, 2005; Chang et al., 2006; Sheh et al., 2007). Heart rate (HR) was estimated instantaneously from the digitized SAP signals. We are particularly interested in the LF (0.25-0.8 Hz) component in the SAP spectrum because it takes origin from the RVLM (Kuo et al., 1997a) and its power density mirrors the prevalence of baroreceptor reflex-mediated sympathetic neurogenic vasomotor discharges that emanate from this brain stem site (Li et al., 2001). More importantly, our laboratory demonstrated previously (Chan et al., 2001a, 2003b, 2004a, 2005d, 2007a; Li et al., 2005; Chang et al.,
2006) that the power density of this spectral signal exhibits triphasic changes that reflect the “pro-life” and “pro-death” phases in our experimental endotoxemia model of brain stem death (Chan et al., 2005b). The power density of the LF component was displayed on-line during the experiment, alongside pulsatile SAP, mean SAP (MSAP) and HR, in a real-time manner.

**Experimental endotoxemia.** *Escherichia coli* LPS (serotype 0111:B4, Sigma; 15 mg/kg) was administered intravenously, with saline serving as the vehicle control. As we reported previously (Chan et al., 2001a, 2003b, 2004a, 2005d, 2007a; Li et al., 2005; Chang et al., 2006), the sequence of cardiovascular events during this LPS-induced endotoxemia can be divided into a reduction (Phase I), followed by an augmentation (Phase II; “pro-life” phase) and a secondary decrease (Phase III; “pro-death” phase) in the power density of the LF component of SAP signals. There was also progressive hypotension, with death ensuing generally within 4 h in approximately 40% of the animals.

**Index for mortality.** We assessed mortality by constructing a survival curve over 4 h after application of LPS. Animals that succumbed to experimental endotoxemia exhibited a dramatic reduction in the power density of the LF component of SAP signals before death (Chan et al., 2002, Li et al. 2005).

**Microinjection of test agents into the RVLM.** To produce site-specific actions, pharmacological agents were microinjected into the RVLM (Chan et al., 2001a,b, 2002, 2003a-c, 2004a,b, 2005a,c, 2007a-c; Li et al., 2001, 2005; Chang et al., 2006; Sheh et al., 2007). The coordinates used were: 4.5 to 5 mm posterior to the lambda, 1.8 to 2.1 mm lateral to the midline and 8.1 to 8.4 mm below the dorsal surface of the cerebellum. The volume of injection was restricted to 50 nL, and possible volume effect of microinjection was controlled by injecting the same amount of solvent. To avoid the confounding effects of drug interactions, each animal received only one test agent.

**Collection of ventrolateral medullary samples.** Since the power density of LF component in the SAP spectrum reflects the prevalence of the “life-and-death” signal detected from patients who suffered from systemic inflammatory response syndrome (Yien et al., 1997), we routinely collected tissue samples (Chan et al., 2001a, 2003a-c, 2004a, 2005d, 2007a; Li et al., 2005; Chang et al., 2006) at the peak of each phase of
experimental endotoxemia (LPS group) or 15 min, 1.5 h or 3 h after i.v. injection of saline (vehicle group). Medullary tissues collected from anesthetized animals but without treatment served as sham-controls. Tissues from both sides of the ventrolateral medulla, at the level of the RVLM (0.5 to 1.5 mm rostral to the obex), were collected by micropunches made with a 1 mm (id) stainless steel bore to cover the anatomical boundaries of the RVLM. The concentration of total or fractional proteins extracted was determined by the BCA Protein Assay (Pierce).

**Western blot analysis.** Western blot analysis was carried out (Chan et al., 2003a,c, 2004a, 2005d, 2007a-d; Li et al., 2005; Chang et al., 2006; Sheh et al., 2007) using an appropriate primary antiserum against the targeted protein. This was followed by incubation with horseradish peroxidase-conjugated IgG, and specific antibody-antigen complex was detected by an enhanced chemiluminescence Western blot detection system (NEN). The amount of protein product was quantified by the ImageMaster Video Documentation System (Amersham), and was expressed as the ratio to β-actin protein.

**Isolation of RNA and real-time polymerase chain reaction (PCR).** Total RNA from the ventrolateral medulla oblongata was isolated with TRIzol reagent (Invitrogen) (Chan et al., 2004a, 2007b; Chang et al., 2006). All RNA isolated was quantified by spectrophotometry and the optical density (OD) 260/280 nm ratio was determined. As in our recent study (Chang et al., 2006; Chan et al., 2007b), reverse transcriptase reaction was performed using a SuperScript Preamplification System (Invitrogen) for the first-strand cDNA synthesis. Real-time PCR analysis was performed by amplification of cDNA using a LightCycler® instrument (Roche). PCR reaction for each sample was carried out in duplicate for all the cDNA and for the GAPDH control. Primers were designed using the sequence information of the NCBI database by Roche LightCycler® probe design software 2.0, and oligonucleotides were synthesized by Genemed Biotechnologies. The primer pairs used for amplification of target genes were, nos II: 5’-TGGAGGTGCTGGAAGAGTT-3’ (forward primer) and 5’-GGAGGAGCTGATGGAGTAGT-3’ (reverse primer); or GAPDH cDNA: 5’-GCCAAAAGGGTCATCATCTC-3’ (forward primer) and 5’-GGCCATCCACAGTCTTCT-3’ (reverse primer).

Fluorescence signals from the amplified products were quantitatively assessed using the LightCycler® software program (version 3.5). Second derivative maximum mode
was chosen with baseline adjustment set in the arithmetic mode. The relative changes in 

\( \text{nos II} \) mRNA expression were determined by the fold-change analysis (Chang et al., 2006; Chan et al., 2007b) in which 

\[
\text{Fold change} = 2^{-\Delta\Delta Ct}, \quad \text{where} \quad \Delta\Delta Ct = (C_{tnos II} - C_{tGAPDH})_{LPS \text{ treatment}} - (C_{tnos II} - C_{tGAPDH})_{\text{sham control}}.
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Note that Ct value is the cycle number at which fluorescence signal crosses the threshold.

**Nuclear protein extraction and electrophoresis mobility shift assay (EMSA).** We employed EMSA (Chan et al., 2004a, 2007c,d) to measure NF-κB DNA binding activity in nuclear proteins pooled from ventrolateral medulla of 5 to 6 rats. The 3’ end of a double-stranded synthetic oligonucleotide probe for NF-κB (5’-AGTGAGGGGACTTTCCCAGG-3’ and 3’-TCAACTCCCCTGAAAGGGTCC-5’) (Chan et al., 2004b, 2007c) was labeled with digoxigenin-11-ddUTP (Roche). DNA and protein complexes resolved on 4% polyacrylamide gels by electrophoresis were detected by chemiluminescence after reacting with an anti-digoxigenin antiserum.

**Statistical analysis.** All values are expressed as mean ± SEM. The relative density of protein expression or fold-change in real-time PCR products induced by LPS was used for statistical analysis. One-way analysis of variance was used to assess group means, followed by the Scheffé multiple-range test for post hoc assessment of individual means. Mortality rate was assessed by the Fisher exact test.

**Results and Discussion**

**Drastic difference in magnitude of expression of NOS II at mRNA and protein levels in the RVLM during experimental brain stem death**

The conceptual premise that both synthesis and degradation of NOS II in the RVLM participate in the brain stem death is grounded in the observation that the magnitude of NOS II expression at mRNA (synthesis) and protein (degradation) levels after the elicitation of experimental endotoxemia was drastically different. As illustrated in Figure 1, the protein expression of NOS II in THE RVLM underwent a progressive increase that amounted to 20% over sham-controls during Phase III. On the other hand, there was an abrupt augmentation of NOS II mRNA during Phases II and III, reaching approximately 8 folds over sham-controls during Phase III endotoxemia.
Synthesis of NOS II in the RVLM by the ubiquitin-proteasome system is involved in the “pro-death” process

Results from EMSA (Figure 2) support the notion that synthesis of NOS II via activation of NF-κB following IκB degradation by the ubiquitin-proteasome system in the RVLM took place during experimental brain stem death. There was a significant and progressive increase in the association of NF-κB with its consensus DNA oligonucleotide in nuclear extracts from the ventrolateral medulla during the 3 phases of experimental endotoxemia. We confirmed that this association was not due to non-specific binding when competitive assay using unlabeled NF-κB oligonucleotide resulted in discernible disappearance of NF-κB DNA binding. Intriguingly, pretreatment by microinjection bilaterally of a non-specific proteasome inhibitor, lactacystin (1 nmol) into the RVLM discernibly antagonized the augmented nucleus-bound translocation of NF-κB. In addition, pretreatment with a specific inhibitor of IκB degradation by the ubiquitin-proteasome system, proteasome inhibitor II (1 nmol) elicited comparable results (Figure 2).

Our laboratory has demonstrated that an upregulation of NOS II through NF-κB activation at the RVLM (Chan et al., 2001a,b, 2003b, 2004a,b, 2007c) plays a pivotal role in the reduction of the “life-and-death” signal in our experimental endotoxemia model during the progression towards brain stem death. It thus is important to show that the identified engagement of the ubiquitin-proteasome system at the RVLM in synthesis of NOS II via activation of NF-κB following IκB degradation is causally related to the “pro-death” process during experimental brain stem death. Figure 3 shows that there was a progressive increase in the number of animals that succumbed to endotoxemia beginning 100 min after administration of LPS at the dose we used (15 mg/kg, i.v.), reaching a mortality rate of approximately 40% within 4 h. Intriguingly, pretreatment with either lactacystin or proteasome inhibitor II, at the same dose (1 nmol) that antagonized nucleus-bound translocation NF-κB, significantly improved the survival rate to 70-75% (Figure 3) by 4 h. Of note is that this antagonism also took place 100 min after administration of LPS, commensurate with the drastic increase in NOS II mRNA (Figure 1).

Ubiquitination in the RVLM is preserved during experimental brain stem death

As reflected by Hershko (1996), the discovery of the ubiquitin-proteasome system was originally driven by the notion that intracellular protein degradation is energy-dependent. It is since known that two ATP-dependent steps are present during
ubiquitination; formation of a high-energy thiolester bond between ubiquitin and the ubiquitin-activating enzyme (E1) and degradation of ubiquitinylated proteins by the 26S proteasome complex (see reviews by Finley and Chau, 1991; Hershko and Ciechanover, 1992; Coux et al., 1996; Ciechanover, 1998; Ciechanover and Schwartz, 1998; Hershko and Ciechanover, 1998; Tanaka, 1998; Voges et al., 1999; Glickman and Ciechanover, 2002). In this context, it is intriguing to note from our experimental endotoxemia model of brain stem death that mitochondrial dysfunction, leading to bioenergetic failure (Chuang et al., 2002, 2003), takes place in the RVLM during the progression towards death. An immediate corollary to these observations is that the process of ubiquitination in the RVLM may be progressively compromised during brain stem death. Figure 4, however, indicated that polyubiquitination detected by immunoblot remained constant throughout the three phases of experimental endotoxemia. Moreover, the magnitude of polyubiquitination was augmented on inhibiting proteasome activities with lactacystin (1 nmol), a conventional index that ubiquitination was preserved in the RVLM.

De-ubiquitination in the RVLM is also preserved during experimental brain stem death

Evidence is accumulating that suggests a functional role for the de-ubiquitinating enzymes (Wilkinson, 1997). Of the known de-ubiquitination enzymes, the ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1) is among the most abundantly present proteins in the brain (Doran et al., 1983). Limited studies on its physiological or pathological roles in the nervous system suggest that deficiency in UCH-L1 may be related to neurological diseases (Lowe et al., 1990; Leroy et al., 1998; Saigoh et al., 1999; Kurihara et al., 2001; Nishikawa et al., 2003; Osaka et al., 2003; Castegna et al., 2004; Choi et al., 2004). It follows that by reducing the availability of ubiquitin for optimal operation of protein degradation by the ubiquitin-proteasome system in the RVLM, a compromise of the de-ubiquitination process may affect the survival of animals that are subject to life-threatening insults such as brain stem death. Results from Western blot analysis again showed that this is not the case. Both the levels of UCH-L1 and monoubiquitin (free form of ubiquitin) remained constant during the three phases of experimental endotoxemia.

De-ubiquitination is crucially involved in the “pro-life” process

Our laboratory reported previously that UCH-L1 in the RVLM is crucial to survival during intoxication induced by the organophosphate poison mevinphos (Chang et al.,
2004). Following this lead, we found in the present study that de-ubiquitination in the RVLM is crucially involved in the “pro-life” process during brain stem death. Pretreatment by microinjection bilaterally into the RVLM of a non-specific inhibitor of de-ubiquitinating enzymes, ubiquitin aldehyde, at a very low dose of 5 fmol, was effective in reducing the survival rate by 30% right from the onset of experimental endotoxemia, with 60% mortality by 80 min (Figure 6). Increasing the dose to 50 fmol further exacerbated the mortality rate to 80% by 20 min, with all animals succumbed to endotoxemia by 100 min. Pretreatment with ubiquitin aldehyde at 5 pmol resulted in 100% mortality within 5-10 min after administration of LPS. Selective inhibition of UCH-LI (1 nmol) also significantly reduced survival rate that began within the first 20 min in animals subject to experimental endotoxemia, leading to a survival rate of 10% at the end of 4 h (Figure 6).

**Degradation of NOS II by the ubiquitin-proteasome system is crucially involved in the “pro-life” process**

The exacerbation of mortality rate by pretreatments with ubiquitin aldehyde or UCH-LI inhibitor that shifted the survival curve downwards and left-wards, a response pattern that is entirely different from pretreatments with lactacystin or proteasome inhibitor II, together with the observations that both ubiquitination and de-ubiquitination in the RVLM were preserved during experimental brain stem death, strongly suggest that degradation of “pro-death” proteins is crucially involved in the “pro-life” process. Our results showed that one of those “pro-death” proteins is NOS II. Figure 7 shows that in the presence of lactacystin, the expression of NOS II protein in the RVLM during Phases I and II was significantly augmented when compared to vehicle control, although there was no further increase during Phase III. We interpret these data to suggest the presence of a balance between degradation of NOS II that takes place throughout our 240-min observation period and synthesis of this isoforms that becomes prominent during Phases II and III. This interpretation is reinforced by results from proteasome inhibitor II pretreatment. By specifically preventing synthesis of NOS II via NF-κB activation, the progressive increase in NOS II protein expression during experimental endotoxemia was completed antagonized (Figure 7).

Animals that received pretreatments with either UCH-LI inhibitor (1 nmol) or ubiquitin aldehyde (50 pmol) and succumbed to LPS within 5-10 min after administration (Figure 8) further demonstrated that this degradation of NOS II is crucially involved in the “pro-life” process. In the absence of significant increase in
NOS II mRNA in the RVLM, the amount of NOS II was augmented by more than 50%.

**Conclusion**

Based on an experimental endotoxemia model and working with a neural substrate that is intimately related to the “life-and-death” process, the present study revealed several roles for the ubiquitin-proteasome system in the RVLM during brain stem death. We found that both ubiquitination and de-ubiquitination processes in the RVLM are preserved. More intriguingly, we identified that one crucial determinant for life and death resides in the balance between degradation of NOS II protein, which is in operation throughout our 240-min observation period, and synthesis of this isoforms via activation of NF-κB following IκB degradation, which becomes prominent during Phases II and III endotoxemia. This conclusion therefore offers a reasonable explanation on the very disparate temporal patterns of augmentation of NOS II mRNA and protein expression in the RVLM during the progression towards brain stem death. This information shall be invaluable to future development of management strategies against fatal eventualities such as brain stem death.

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**Figure 1.** Temporal changes in NOS II protein expression detected by Western analysis (upper diagram) or NOS II mRNA detected by real-time PCR (lower diagram) in the ventrolateral medulla of rats that received i.v. administration of LPS (15 mg/kg). In this and subsequent figures, C denotes sham-controls, I, II or III denotes Phases I, II or III endotoxemia. Values are mean ± SEM of quadruplicate analyses from 7-8 animals per experimental group. *P < 0.05 versus sham-control group in the Scheffé multiple-range test.

**Figure 2.** Representative gels depicting NF-κB DNA binding in nuclear extracts from the ventrolateral medulla during three phases of experimental endotoxemia in animals that received pretreatment by microinjection bilaterally into the RVLM of lactacystin (Lacta; 1 nmol) (upper diagram), proteasome inhibitor II (PiII; 1 nmol) (lower diagram) or double distilled water (ddH2O) (upper and lower diagrams). A competitive assay with the addition of 100-fold of unlabeled NF-κB oligonucleotide was used to control for non-specific binding (Cold).
**Figure 3.** Survival rate of rats that received pretreatment by microinjection bilaterally into the RVLM of lactacystin (1 nmol; upper diagram), proteasome inhibitor II (1 nmol; lower diagram) or ddH₂O (upper and lower diagrams), followed by i.v. administration of saline or LPS (15 mg/kg). Each group contains 7-8 animals at the beginning of the experiment.

**Figure 4.** Temporal changes in polyubiquitination detected by Western analysis (upper diagram) in the ventrolateral medulla of rats that received i.v. administration of LPS (15 mg/kg). These results are duplicated in the lower diagram showing the effects of pretreatment with lactacystin (1 nmol). Values are mean ± SEM of quadruplicate analyses from 7-8 animals per experimental group. *P < 0.05 versus LPS+ddH₂O group in the Scheffé multiple-range test.
Figure 5. Temporal changes in UCH-L1 (upper diagram) or monoubiquitin (lower diagram) detected by Western analysis (upper diagram) in the ventrolateral medulla of rats that received i.v. administration of LPS (15 mg/kg). Values are mean ± SEM of quadruplicate analyses from 7-8 animals per experimental group. No significance exists among all groups (P > 0.05) in one-way ANOVA.

Figure 6. Survival rate of rats that received pretreatment by microinjection bilaterally into the RVLM of ubiquitin aldehyde (UbAd; 5 fmol, 50 fmol or 5 pmol) (upper diagram), ubiquitin carboxyl-terminal hydrolase isozyme L1 inhibitor (UCH-L1i; 1 nmol; lower diagram) or ddH2O (upper and lower diagrams), followed by i.v. administration of saline or LPS (15 mg/kg). Each group contains 7-8 animals at the beginning of the experiment.
**Figure 7.** Temporal changes in NOS II protein expression detected by Western analysis (upper diagram) in the ventrolateral medulla of rats that received pretreatment by microinjection bilaterally into the RVLM of lactacystin (1 nmol; upper diagram), proteasome inhibitor II (1 nmol; lower diagram) or ddH2O (upper and lower diagrams), followed by i.v. administration of LPS (15 mg/kg). Values are mean ± SEM of quadruplicate analyses from 7-8 animals per experimental group. *P < 0.05 versus sham-control group, and †P < 0.05 versus LPS+ddH2O group in the Scheffé multiple-range test.

**Figure 8.** NOS II protein expression detected by Western analysis (upper diagram) or NOS II mRNA detected by real-time PCR (lower diagram) in the ventrolateral medulla of rats that received pretreatment by microinjection bilaterally into the RVLM of UCH-L1 inhibitor (1 nmol), ubiquitin aldehyde (50 fmol), artificial cerebrospinal fluid (aCSF) or DMSO, and died within 5-10 min following i.v. administration of LPS (15 mg/kg). Values are mean ± SEM of quadruplicate analyses from 7-8 animals per experimental group. *P < 0.05 versus aCSF group in the Scheffé multiple-range test in the upper diagram. No significance exists among all groups (P > 0.05) in one-way ANOVA in the lower diagram.
Figure 1

(NOS II protein)

(NOS II mRNA)

Figure 1
Figure 2
Figure 3
Figure 4
Figure 5

UCH-L1 / β-actin (%)

monoubiquitin / β-actin (%)

CII I I I I

C I II III

C I II III
Figure 6
Figure 7
Figure 8

(a) Relative amount of NOS II mRNA

(b) NOS II / β-actin (Fold)

C aCSF 40% DMSO UCH-L1i UbAD

LPS

*