Induction of NADPH diaphorase/nitric oxide synthase in the spinal cord motor neurons of rats following a single and multiple non-penetrative blasts

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Summary. The present study has demonstrated the induction of nicotinamide adenine dinucleotide phosphate-diaphorase (NADPH-d) reactivity and nitric oxide synthase-like immunoreactivity (NOS-LI) in the ventral horn motoneurons of the spinal cord in rats subjected to a single or multiple underground, or a single surface blast. Both enzyme activities were first detected in some motoneurons in laminae VIII and IX of Rexed, 3 hours after the blast. Some NADPH-d and NOS-LI positive neurons were also distributed in laminae VI and VII. The number and intensity of the labelled cells appeared to increase progressively, peaking at 2-3 days after the blast but were drastically reduced thereafter, so that at 7 days after the blast only a few positive neurons were observed. In rats killed at 2 weeks and in longer surviving intervals, i.e. up to 1 month, NADPH-d/NOS reactivity in the ventral horn motor neurons had diminished. The functional significance of the transient expression of neuronal NADPH-d/NOS after the blasts remains uncertain, although from a speculative point of view, the induction of these enzymes probably would reflect an increased production of nitric oxide (NO). In view of the lack of atrophic changes in most, if not all, of motor neurons, it is suggested that the increased levels of NO production after the blast injury may be involved in a neuroprotective function.

Key words: NADPH-d, Nitric oxide synthase, Blast, Spinal cord neurons, Rat

Introduction

Neurons containing nitric oxide (NO) have been identified histochemically by the presence of nicotinamide adenine dinucleotide phosphate diaphorase (NADPH-d) or immunocytochemically by the presence of nitric oxide synthase (NOS), the enzyme responsible for NO synthesis. A one-to-one correlation between NADPH-d positive neurons and NOS immunoreactive neurons has been reported in different areas of the central nervous system (Bredt et al., 1991; Dawson et al., 1991). Therefore, the localization of NADPH-d is generally considered to be reflective of the presence of NOS and, hence, its use as a specific histochemical marker for neurons containing NO.

Very interestingly NADPH-d containing neurons were found to survive some degenerative processes, e.g. Huntington’s (Ferrante et al., 1985) and Alzheimer’s diseases (Kowall and Beal, 1988) in select areas, and in hypoxic-ischaemic brain injury (Uemura et al., 1990); furthermore, they were resistant to various neurotoxins (Koh et al., 1986), including excitatory amino acids (Beal et al., 1986; Koh et al., 1986). In the rat spinal cord, NOS immunoreactivity is described to be absent in the ventral horn motoneurons (Dun et al., 1992), although recent study (Dun et al., 1993) stated that a few NOS-immunoreactive neurons were detected in the similar region. However, NADPH-d activity was induced in motoneurons following ventral root avulsion (Wu, 1993; Wu and Li, 1993; Wu et al., 1993, 1995) and urethral obstruction (Zhou and Ling, 1997). Similar induction of NADPH-d activity has been reported in the vagal motoneurons after vagotomy (Gonzalez et al., 1987; Jia et al., 1994) and in moto-neurons of cranial nerves after axotomy (Yu, 1994).

The aim of this study was to examine the expression of NADPH-d/NOS, if any, in the spinal motoneurons innervating the limb muscles in rats subjected to non-penetrative blast. This is because our recent studies (Kaur et al., 1995, 1997) have reported atrophic changes ultrastructurally in the dendrites of cortical and cerebellar neurons as well as widespread activation of microglial cells, a hallmark of neurodegeneration, after a single blast in rats. This information would be useful for better understanding of the effects of blast on the structural integrity of the central nervous system at the spinal level and consequently on the motor activity of
limb muscles. This would provide great potential for therapeutic interventions in the event of undesirable blast injuries in military training and exercises.

Materials and methods

Seventy-two male Wistar rats (200-250g) subjected to blasts and 26 normal rats were used in this study. The rats subjected to the blasts were deeply anaesthetized with 7% chloral hydrate before being transported to the blast site. They were divided into 3 groups. Group I consisted of 30 rats which were subjected to a single non-penetrative blast. The rats were kept in separate laboratory rat cages (North Kent, England) (each contained 8-10 rats) secured to the floor in an underground concrete chamber simulating a bunker shelter as was reported previously (Kaur et al., 1995, 1997). The explosive (nitrate based conventional explosive, TNT compound B; 110kg TNT equivalent) was then detonated underground 3.5 meters below the ground surface next to the chamber separated by a concrete wall. The combination of explosive size and distance between explosive charge and animals were based on other considerations, including strength and stability of the infrastructure as had been determined previously. Group II consisted of 12 rats which were subjected to multiple (double or triple) underground blasts on alternate days. Group III consisted of 30 rats, subjected to a single surface blast. In this instance, the rats were kept in two separate laboratory rat cages placed on the ground surface, located at 50m (n=15) and 100m (n=15) away from the explosive charge. Six rats situated 50m away from the explosive charge, died immediately or overnight following the blast. The surviving rats from the above groups were then returned to the animal house in the laboratory within 2 hours and were killed at various time intervals ranging from 3 hours to 1 month after the blast. In the handling and care of all animals, the international guiding principle for animal research as stipulated by WHO Chronicle (1985) and as adopted by the Laboratory Animal Centre, National University of Singapore, were followed.

For NADPH-d histochemistry, 30 rats subjected to the blast were deeply anaesthetized with 7% chloral hydrate and killed at various time intervals after the blast as for NADPH-d histochemistry. Two control rats were killed at each of the corresponding interval. They were perfused with Ringer’s solution until the liver and lungs were clear of blood. This was followed by an aldehyde fixative composed of a mixture of periodate-lysine-parafomaldehyde with a concentration of 2% parafomaldehyde. Following the perfusion, the spinal cord at C6-C7 and L6-S1 segments was removed and kept in a similar fixative for 2 hours. They were then kept in 0.1M phosphate buffer containing 10% sucrose overnight at 4 °C. Frozen coronal sections of the spinal cord of 40µm thickness were cut and rinsed in phosphate-buffered saline (PBS). The sections were then incubated in the primary antibody directed against brain NOS (a rabbit polyclonal anti-NOS, Transduction Lab, USA) with a dilution of 1:70 with PBS. Subsequent antibody detection was carried out by using Vectastain ABC kit (PK 4001, Vector Lab.) against rabbit IgG with 3,3' -diaminobenzidine tetrachloride (DAB) as a peroxidase substrate.

For Nissl staining, 2 normal and 6 rats subjected to single or surface blast were deeply anaesthetized with 7% chloral hydrate and killed at 2 (n=2), 3 (n=2) weeks and 1 (n=2) month after the blast. The rats were perfused with Ringer’s solution followed by 10% neutral formalin. After perfusion, the spinal cord at C6-C7 and L6-S1 levels was removed and kept overnight at 4 °C in the same fixative. The spinal cord segments were then dehydrated in an ascending series of alcohol, cleared with toluene and embedded in paraffin wax. 7µm thick transverse serial sections were cut and stained with cresyl fast violet.

Results

NADPH-d histochemistry

Normal (control) animals

In the spinal cord at cervical and lumbar segments of the normal (Figs. 1, 2) rats, NADPH-d positive neurons and fibres were located mainly in the dorsal horns (laminae I to IV of Rexed) and the central gray (lamina X) immediately surrounding the central canal (Figs. 1, 2). A few NADPH-d positive neurons also occurred in the lateral horn (lamina VII) at the lower lumbar segments. In general, NADPH-d positive neurons were absent in the bilateral ventral horns of the spinal cord.
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Single (underground) blast

All surviving rats regained full consciousness on returning to the animal house from the blast site. They appeared physically healthy with no apparent motor deficits at the various time intervals at which they were killed after the blast.

There was no difference in NADPH-d reactivity of neurons in the dorsal horns and the gray matter around the central canal compared with cells of the normal rats. A noticeable difference, however, was the appearance of a variable number of NADPH-d positive neurons in the bilateral ventral horns (laminae VIII and IX); some NADPH-d positive neurons were also distributed in laminae VI and VII. NADPH-d reactivity was first detected in a few motoneurons in the ventral horn, 3 hours after the blast (Figs. 3, 4). The number and intensity of the labelled cells appeared to increase with time, so that at 2 days after the blast many labelled cells were observed (Figs. 5, 6). The NADPH-d staining of the bilateral ventral horn motoneurons at C6-C7 and L6-S1 segments was comparable. In general, the induced

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**Fig. 1.** Transverse section through the cervical enlargement of the spinal cord of a normal rat stained for NADPH-d. Sporadic NADPH-d positive neurons (arrows) are distributed in the dorsal horns and the gray matter (lamina X) around the central canal but are undetectable in the ventral horns. Roman numerals denote laminae of Rexed. Bar: 250 μm.

**Fig. 2.** Transverse section through the lumbar enlargement of the spinal cord of a normal rat stained for NADPH-d. Arrows indicate some NADPH-d positive neurons in the gray matter (lamina X) around the central canal. Roman numerals denote laminae of Rexed. Bar=250 μm.

**Fig. 3.** Transverse section through the cervical enlargement of spinal cord, 3 hours after a single (underground) blast. Moderate NADPH-d stained neurons (arrows) are observed in laminae (Rexed) IX in the ventral horns, and the area (lamina X) immediately surrounding the central canal. Bar: 250 μm.

**Fig. 4.** Transverse section through the lumbar enlargement of spinal cord, 3 hours after a single (underground) blast. NADPH-d reactivity is detected in a few neurons (arrows) in laminae (Rexed) VIII and IX in the ventral horns, and the area (lamina X) immediately surrounding the central canal. Bar: 250 μm.
NADPH-d reactivity in the motoneurons in the ventral horn was lighter than that of NADPH-d positive neurons normally existing around the central canal and in the dorsal horn. The reaction product was deposited mainly in the neuronal perikarya and processes. The number of NADPH-d positive ventral horn motoneurons appeared to decline at 7 days showing only a few NADPH-d positive cells. In rats killed at 2 weeks and in longer surviving intervals, NADPH-d positive neurons were absent in the ventral horn but persisted in the dorsal horn and the central gray.

Multiple (underground) blasts

In rats subjected to multiple blasts and killed at 3 hours after the final blast, a large number of neurons in the ventral horn in both the cervical and lumbar segments of the spinal cord was induced to express NADPH-d activity (Figs. 7-9). The number of NADPH-d reactive motoneurons was greater than in rats receiving a single blast and killed at the corresponding time intervals. As in animals subjected to a single blast, induced NADPH-d positive neurons were distributed mainly in laminae IX, VIII and VII (Figs. 7, 8).

Surface blast

Except for rats located at 100 m from the explosive charge, all surviving rats at 50 m remained deeply unconscious when returned to the animal house from the blast site. It was only after a few hours had elapsed that the rats started to regain consciousness. On recovery, the rats appeared lethargic and weak. On the other hand, rats located at 100 m from the explosive charge recovered readily as in rats after a single (underground) blast.

The results in rats subjected to surface blast paralleled those after multiple blasts. NADPH-d positive neurons were conspicuous in the ventral horn of rats situated 50 m away from the explosive charge (Figs. 10-12). In rats situated 100 m away from the explosive charge, NADPH-d positive neurons were not observed in the ventral horn.

NOS immunohistochemistry

In normal rats, some neurons in the dorsal horn and gray matter around the central canal were labelled for NOS. The motoneurons in the ventral horn did not show any detectable NOS immunoreactivity.

Following the underground single or multiple blasts, besides some positive neurons in the dorsal horn and around the central canal, a variable number of motoneurons in the ventral horns were induced to express NOS-like immunoreactivity (NOS-LI). The number and intensity of immunoreaction of labelled neurons increased progressively after the blast so that a large number of motoneurons showing intense NOS-LI were observed between 3 hours to 3 days after the blast (Figs. 13, 14). The number and intensity of immunoreaction of labelled neurons appeared to decline at 5-7 days so that in rats killed at longer time intervals NOS positive motoneurons were absent.

Following the surface blast, the NOS-LI in motoneurons in the ventral horns was comparable to that of the underground blasts. Furthermore, it paralleled the NADPH-d reactivity in rats especially those placed at 50 m from the explosive charge (Figs. 15, 16). In rats placed at 100 m NOS-LI immunoreactivity was undetectable in the motoneurons of the ventral horns.

Nissl staining

All neurons of the spinal cord in normal or rats subjected to different blasts appeared structurally normal. Occasional hyperchromatic cells, however, were observed in the ventral horns after the blast (Figs. 17, 18).

Discussion

The present study has demonstrated the induction of NADPH-d/NOS in motoneurons in the ventral horn of the rat spinal cord following different modes of non-penetrative blast. This appeared to be selective since the NADPH-d/NOS positive neurons which normally exist around the central canal and in the dorsal horn remained...
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unaffected, suggesting the greater sensitivity of the ventral horn motoneurons to the blast force. A similar induction in NADPH-d activity has been reported in motoneurons in the lumbar spinal cord after ventral root avulsion (Wu, 1992), and urethral obstruction (Zhou and Ling, 1997). Although Dun et al. (1993) reported the
presence of a few NOS-immunoreactive neurons in the rat spinal cord ventral horn, NOS immunoreactivity has been generally accepted to be absent in the motoneurons in this region (Dun et al., 1992; Zhou and Ling, 1997). Present results confirm the plasticity of NADPH-d/NOS in the motoneurons of spinal cord. Since NADPH-d/NOS reactivity was not observed in motoneurons of the ventral horn in control rats, it can be confidently concluded that it was attributed to the blast force. The underlying mechanisms leading to the expression of NADPH-d/NOS in the motoneurons of the spinal cord remain purely speculative. We have reported previously (Kaur et al., 1995) that a single non-penetrative blast could cause widespread damage in the brain in which some neurons in the cerebral cortex displayed signs of atrophy affecting their dendrites. It is possible that some of the injured neurons may represent motoneurons projecting to the spinal cord. Since neurons in injury such as ischaemia are described to release glutamate (Rothman and Olney, 1987; Dragunow et al., 1990; Colllaco et al., 1994) and since glutamatergic neurotransmission through the corticospinal tracts and excitatory interneuronal pathways in the spinal cord has been reported by others (Young et al., 1983; O'Brien and Fischbach, 1986) it is possible that release of glutamate by the corticospinal neurons at their terminals in the spinal cord may have elicited the activation of N-methyl-D-aspartate (NMDA) receptors on the postsynaptic motoneurons which would conceivably lead to subsequent increase in intracellular calcium levels and

Fig. 10. Transverse section through the cervical enlargement of spinal cord, 3 hours after a single surface blast of rat situated 50 m away from the explosive charge. NADPH-d positive neurons (arrows) are distributed in laminae VI, VII and IX. Bar: 250 μm.

Fig. 11. Enlarged view of NADPH-d stained motoneurons (arrows) in the ventral horn of Fig. 10. Bar: 50 μm.

Fig. 12. Enlarged view of Fig. 10. showing NADPH-d stained motoneurons (arrows) in the ventral horn of the cervical spinal cord. Bar: 50 μm.
Fig. 13. Transverse section through the cervical enlargement of spinal cord, 1 day after multiple (triple) underground blasts. NOS positive neurons (arrows) are distributed in the ventral horn. Bar: 250 µm.

Fig. 14. Enlarged view of NOS positive neurons (arrows) in the ventral horn of spinal cord 1 day after multiple (triple) underground blasts. Bar: 50 µm.

Fig. 15. Transverse section through the cervical enlargement of spinal cord, 3 hours after a single surface blast of a rat situated 50 m from the explosive charge. NOS positive neurons (arrows) can be seen in the ventral horn. Bar: 250 µm.

Fig. 16. Enlarged view of NOS positive neurons (arrows) in the ventral horn of spinal cord 3 hours after surface blast (50 m). Bar: 50 µm.

Fig. 17. Nissl stained preparations of transverse section through the lumbar enlargement of spinal cord, 12 days after a single (underground) blast. Virtually all motoneurons in the ventral horn appear structurally normal except for one darkened cell (arrow) in lamina VIII. Bar: 250 µm.

Fig. 18. Enlarged view of a Nissl stained darkened cell (arrow) in the ventral horn of Fig. 17, 12 days after a single (underground) blast. Bar: 50 µm.
activation of NOS.

The significance of induced expression of NADPH-d and NOS in blast injury remains uncertain. NO has been implicated in a variety of functions including the nonadrenergic noncholinergic (NANC) neurotransmission in the peripheral nervous system. However, when present in high concentrations, NO may be involved in glutamate neurotoxicity mediated by NMDA receptors and is responsible for neuronal death in culture (Dawson and Dawson, 1996). Recent studies also indicate that NO may possess neuroprotective properties either by inhibition of NMDA receptor function (Lei et al., 1992; Manzoni et al., 1992) or blocking free radical damage (Nathan, 1992). It is also reported that NO possesses both neuroprotective/neurodestructive properties depending upon the redox milieu. The NO radical (NO•) is neurodestructive, while NO+ (i.e. NO complexed to a carrier molecule) is neuroprotective (Lipton et al., 1993). NOS expressed in injured motoneurons is thought to signal the impending death of injured cells or to act as a killer protein that produces neurotoxic levels of NO. It has been shown that spinal root avulsion caused induction of neuronal NOS in motoneurons which was coincident with the death of the injured motoneurons (Wu, 1993; Wu and Li, 1993; Wu et al., 1993, 1995). However, other results have been contradictory which show no evidence of NO-mediated neuronal death in vivo (Pauwels and Leysen, 1992; Rose and Choi, 1992). Gonzalez et al. (1987) reported that neuronal NADPH-d staining in the dorsal motor nucleus of the vagus was increased after cervical vagotomy and suggested that the increase was likely to be linked to a regenerative response. NADPH-d containing neurons were also found to survive the degenerative processes of Huntington’s and Alzheimer’s disease, for example, up to 95% of striatal neurons degenerate, while virtually all NADPH-d containing striatal neurons survive (Ferrante et al., 1985). In the present study, the intense NADPH-d/NOS staining after blast injury is probably necessitated for higher levels of NO production for neuroprotection, since all ventral horn motoneurons containing the enzymes appeared structurally normal. The occasional occurrence of hyperchromatic cells suggesting atrophy could have been produced by fixation artefact as darkened neurons have been reported even in normal neural tissues (Cammermeyer, 1962; Mugnaini, 1965).

At an early time interval (3 hours) after a single blast, very few NADPH-d/NOS stained neurons occurred in the ventral horn of the spinal cord. However, the number as well as the intensity appeared to increase at 2-3 days but was reduced from 5-7 days onwards suggesting the reversible nature of the blast effect. Another feature worthy of note is that the number of NADPH-d/NOS positive motoneurons in rats receiving underground multiple blasts or surface blast was greater than in rats receiving a single underground blast and killed at corresponding time intervals. This suggests the cumulative effects of the blast force. The intensity of NADPH-d reactivity and NOS immunoreactivity of both cervical and lumbar ventral horn neurons was comparable suggesting that the cells are equally susceptible to the blast wave.

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